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Baicalein mediates the anti-tumor activity in Osteosarcoma through lncRNA-NEF driven Wnt/β-catenin signaling regulatory axis

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ABSTRACT

Background: Osteosarcoma (OS) is a common type of malignant bone tumor in adolescents with high risk of metastasis. However, the clinical management still remains unsatisfactory. Traditional Chinese medicine (TCM) has been widely considered as an alternative treatment, and their extracts have proved to possess great potential for drug discovery. Baicalein (BA), the active pharmaceutical ingredient of *rhizoma coptidis*, was proved to have anti-tumor properties in OS, but the mechanism remains poorly understood.

Methods: The potential anti-cancer effects on cell growth, cell cycle, apoptosis and migration were examined in OS cells. Moreover, the lncRNA-Neighboring Enhancer of FOXA2 (lncRNA-NEF) and Wnt/β-catenin signaling were detected by qPCR and Western blotting assays. The *in vivo* effect of GA on tumor growth was investigated using a xenograft mice model.

Results: In the present study, BA was found to significantly suppress tumor growth *in vitro* and *in vivo*. And it was also found to inhibit the invasion and metastasis as well. As for the mechanism investigation, lncRNA-NEF was obviously upregulated by BA in OS cells, and thus induced the inactivation of Wnt/β-catenin signaling. Moreover, lncRNA-NEF knockdown partially reversed the BA-induced anti-cancer activities; and successfully compensated the suppressive effect on Wnt/β-catenin signaling. We therefore suggested that BA induced the inactivation of Wnt/β-catenin signaling through promoting lncRNA-NEF expression.

Conclusions: In conclude, our results demonstrated that BA suppressed tumor growth and metastasis *in vitro* and *in vivo* through an lncRNA-NEF driven Wnt/β-catenin regulatory axis, in which lncRNA-NEF was upregulated by BA, and thus induced the inactivation of Wnt/β-catenin signaling.

The Translational potential of this article: The findings derived from this study validates the anti-cancer activity of BA in OS and provides a novel underlying mechanism, which suggest that BA may be a potential candidate to develop the effective drug for OS patients.

1. Introduction

Osteosarcomas (OS) are the most frequent primary bone sarcomas, affecting mainly children and adolescents. They often locate in the long bone including the distal femur and proximal tibia. Current treatments for OS patients typically include surgery in combination with adjuvant

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and neo-adjuvant chemotherapeutic agents [1,2]. However, OS are very heterogeneous tumors both at the intra- and inter-tumor level, which makes the OS patients with high risk of metastasis and recurrence. The clinical management still remains insufficient, and the patient survival has not improved significantly in recent decades [3,4]. It is therefore urgently to identify a more effective therapeutics strategy for OS patients.

Traditional Chinese medicine (TCM) has been practiced for thousands of years and is widely accepted as an alternative treatment for cancer. Many herbs have been reported to exhibit anti-cancer activity, and their extracts have proved to possess great potential for drug discovery. A number of TCM phytochemicals have been reported to play anti-tumor roles in OS [5,6]. Baicalein (BA) is a flavonoid compound derived from the root of *Scutellaria baicalensis* which has historically been used to protect against oxidant, virus, bacteria, inflammation and allergy [7,8]. Recently, BA has been found to own anti-cancer activities in various cancers, and it induced apoptosis and inhibited metastasis in OS [9–11]. However, the underlying mechanism remains sketchy and sparse.

A large number of studies in the past decade have changed our perspective of ncRNA from junk transcriptional products to vital roles that regulate cellular processes, with extensive literature confirming that ncRNA are involved in chromatin remodeling, transcription, post-transcriptional modification, and signal transduction. As two important members of the ncRNA family, a large number of studies have confirmed that miRNAs and lncRNAs are involved in regulating a variety of biological activities and human disease progression. For example, microRNA-378, microRNA-218, and lincROR have been confirmed to participate in osteogenesis [14–16]. There is mounting evidence confirming that lncRNAs constitute an important component of tumor biology, and several lncRNAs such as H19, Hotair have been identified to modulate cell proliferation, apoptosis, and metastasis in tumorgenesis [17]. LncRNA-Neighboring Enhancer of FOXA2 is one recently identified lncRNA, and our group firstly named it as lncRNA-NEF. Our previous study demonstrated that this lncRNA antagonized epithelial to mesenchymal transition (EMT) and cancer metastasis in hepatocellular carcinoma (HCC) [18]. During last three years, dozens of papers reported this lncRNA mediated tumor growth and metastasis in various cancers such as breast cancer [19], cervical carcinoma [20], esophageal carcinoma [21], gastric carcinoma [22], lung cancer [23], etc. Wnt/β-catenin signaling was also demonstrated to participate in this lncRNA mediated tumorigenesis and metastasis [18]. Especially in OS, lncRNA-NEF was found to be down-regulated, and its overexpression inhibited cancer cell migration and invasion by suppressing miRNA-21 [24].

In this study, BA was confirmed to exhibit anti-tumor activity against OS *in vitro* and *in vivo*. Regarding to the investigation of underlying mechanisms, our results showed that lncRNA-NEF was obviously up-regulated by BA in OS cells, and thus led to the inactivation of Wnt/β-catenin signaling. Therefore, BA suppressed tumor growth and metastasis in OS *via* the lncRNA-NEF driven Wnt/β-catenin regulatory axis. The findings obtained from this study suggest that BA may a potential candidate to develop the effective drug for OS patients.

2. Materials and methods

2.1. Preparation of BA

Baicalein was purchased from Aladdin (Shanghai, China) with purity over 99%. This drug was dissolved in DMSO and stocked at −20 °C for usage.

2.2. Cell culture and treatment

Three OS cell lines including 143 B, MG63 and U2OS were cultured in DMEM (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (P/S, Hyclone, Pasching, Austria) in humidified incubator at 37 °C with 5% CO2.

2.3. Cell proliferation assays

The OS cells were seeded in 96-well microplates at the density of 3000 cells per well. They were treated with various concentrations of baicalein for 24, 48, and 72 h, and then 10 μl methylthiazoletetrazolium (MTT, beyotime, Shanghai, China) solution (5 mg/ml) was incubated for another 4 h. The medium was removed, and 100 μl dimethylsulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was measured at 570 nm with Multiskan FC plate reader (Thermo Scientific, USA). The DMSO (0.1%) was used as the control.

2.4. Colony formation assay

Cells were seeded in 6-well microplates at a density of 300 cells per well, and were treated with baicalein for 24 h. Then, they were maintained for another two weeks in baicalein-free medium. Colonies were fixed with methanol and stained with crysital violet stain, and the numbers of colonies were counted by ImmunoSpot analyzers (CTL).

2.5. Flow cytometry examination

Cells were treated with 50 μM baicalein for 48 h, and harvested for flow cytometry examination. The cells were resuspended and stained by the Cell Cycle Detection Kit (KeyGEN, Nanjing, China) for cell cycle examination. On the other hand, the cells were stained with the cell Apoptosis PI Detection Kit (KeyGEN, Nanjing, China) for apoptosis analyses.

2.6. Wound healing assays

The cells were cultured in 6-well and allowed to reach confluence. The scratch was made using a 1 ml sterile pipette tip across the cell monolayer. Media was removed and the cells were incubated with 50 μM baicalein in DMEM supplemented with 1% FBS for another 24 h, and images were captured under a microscope. The migration rate was calculated following the formula: migration rate (%) = (original width - final width)/original width × 100%. All experiments were performed in triplicate.

2.7. Transwell invasion assays

The transwell membranes were purchased from Corning Inc (New York, NY, USA). Transwell invasion assays were performed according to the manufacturer's protocol. Briefly, 1.0×10^4 baicalein-treated cells and control cells were resuspended in serum-free medium and seeded in the upper chamber, which was coated with Matrigel (Corning, USA). The low chambers were filled with the complete DMEM containing 10% FBS. After incubation for 48 h, cells on the upper surface were removed and the membrane were fixed with methanol for 20 min. Then the membrane was stained with 0.1% crystal violet, and images were captured by Multifunctional Cell Imaging Microplate reader (BioTek, USA). The invaded cells were counted in five different fields under microscopy.

2.8. RNA extraction, reverse transcription, and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using Animal Total RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA), and cDNA was reversely transcribed from RNA by PrimeScript™ RT Reagent Kit (TaKaLa, Japan). Quantitative PCR reactions were set up in triplicates and conducted on a LightCycler 480 system (Roche, Basel, Switzerland) using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher, USA) according to the manufacturer's protocol. Relative expression levels of candidate genes were calculated *via* $2^{-\Delta\Delta Ct}$ method and normalized to the housekeeping gene GAPDH. The primer sequences for real-time PCR were listed in Table 1.

Table 1
Primers for qRT-PCR.

Name	Primer sequences used for RT-PCR
lncRNA-NEF_F	CTGCCGTCTTAAACCAACCC
lncRNA-NEF_R	GCCCAAACAGCTCCTCAATT
β-catenin_F	CCGTTCGCCTTCATTATGGA
β-catenin_R	GGCAAGGTTTCGAATCAATCC
GAPDH_F	ACTTTGGTATCGTGGAAGGACTCAT
GAPDH_R	GTTTTTCTAGACGGCAGGTCAGG
MMP2_F	GAGTGCATGAACCAACCAGC
MMP2_R	AAACTTGCAGGGCTGTCCTT
MMP9_F	TCTATGGTCCTCGCCCTGAA
MMP9_R	TTGTATCCGGCAAACTGGCT
N-cadherin_F	GACAATGCCCCTCAAGTGTT
N-cadherin_R	CCATTAAGCCGAGTGATGGT
Vimentin-F	GACGCCATCAACACCGAGTT
Vimentin-R	CTTTGTCGTTGGTTAGCTGGT
CCND1_F	CTGGAGGTCTGCGAGGAACA
CCND1_R	CCTTCATCTTAGAGGCCACGAA
CCND2_F	GAGCCGGACCTAATCCCTCA
CCND2_R	CGGTGCAGCGTCTAGGG
OCT3/4_F	TCGAGAACCGAGTGAGAGGC
OCT3/4_R	CACACTCGGACCACATCCTTC
Survivin_F	ATTTGAATCGCGGGACCC
Survivin_R	GAGAAAGGGCTGCCAGGC
CD44_F	CAGCACCATTTCAACCACAC
CD44_R	GTTGCCAAACCACTGTTCCT
Name	siRNA sequences
silncRNA-NEF-1	GGAGCUGUUUGGGCAAUAATT
silncRNA-NEF-2	GGCACAACGAUCAAAUUCUTT

2.9. Western blotting

Total protein was extracted using the Lysis Buffer (Thermo Fisher Scientific). The nuclear and cytoplasmic fractions were isolated by the Nuclear and Cytoplasmic Protein Extraction Kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. The supernatant fraction was collected by centrifugation and the protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein were separated by 10% SDS-PAGE (Epizyme, Shanghai, CHN) at 120 V for 80min, and then transferred electrophoretically to a nitrocellulose membrane at 100 V for 90min. The transferred membranes were blocked with 5% skim milk for 1 h and then incubated with primary antibody including β-catenin (1:2000; Cell Signaling Technology, USA), Lamin B1 (1:2000; Cell Signaling Technology, USA) and GAPDH (1:2000; Cell Signaling Technology, USA) at 4 °C for 12 h. The membrane was subsequently incubated with second antibody for 1 h, and exposed by the FluorChem R system (ProteinSimple, San Jose, CA, USA) for chemiluminescence. LaminB1 and GAPDH were used as the internal control of nucleic protein and total protein, respectively.

2.10. Cell transfection

Specific small interfering RNA for lncRNA-NEF (siNEF) was designed and synthesized by TSINGKE Biological Technology (Beijing, China). OS cells were seeded and transfected by Lipofectamine 3000 (Invitrogen) within 24 h.

2.11. Luciferase activity assays

Cells were seeded in 24-well plates, and the luciferase reporter TOPflash was transfected into cells by Lipofectamine 3000 (Invitrogen). Twelve hours later, cells were incubated with GA for 48 h, and then lysed and subjected to firefly luciferase activity assays by the Bright-Glo™ Luciferase Assay System (Promega, Madison, WI, USA) on a Hybrid Multi-Mode Microplate Reader. The renilla luciferase activity was used to normalize the firefly luciferase activity. All the experiments were performed in triplicates.

2.12. Osteosarcoma intra-tibia tumor-bearing model

According to previous reports, the intra-tibia tumor-bearing model was chosen for the *in vivo* experiments [25]. Female Balb/c-nude mice (4–5 weeks old) were purchased from the Laboratory Animal Center, Southern Medical University. All experimental procedures were approved by the Ethics and Animal Research Committee of Southern Medical University Guangzhou, China. MG63 cells (1×10^6/100 μl) were injected into the medullary cavity of the right tibia of mice. When the tumor volume was visible, animals were randomly assigned to two groups (n = 6). Group 1 was intraperitoneally injected BA (40 mg/kg), and group 2 was administrated with vehicle solution buffer (10%DMSO + 40%PEG300 + 5%Tween-80 + 45%saline) with same volume as group 1. BA was administrated every day, and tumor size was measured every 2 days. Tumor volume was calculated according to the following formula: volume=$(W1 \times W2^2)/2$, where W1 = major diameter(mm) and W2 = minor diameter(mm) of tumors.

2.13. Histological examination

Tumor specimens were fixed in 4% paraformaldehyde overnight and then embedded in paraffin. The tumor sections were incubated with the Ki-67 antibody (Calbiochem, Darmstadt, Germany) and β-catenin antibody with 1:50 dilution for 4 h. Visualization was achieved by using the 3, 3′-diaminobenzidine substrate (Dako, Denmark) followed by counterstaining with hematoxylin. The representative images were taken with × 40 magnification and the positive cells were quantified with Image J.

2.14. Statistical analysis

All the experiments were repeated three times, and the experimental data were expressed as the means ± SD. Difference between two independent groups were compared by using Student's t-test and two-way ANOVA. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. BA significantly inhibited cell viability in OS cells

To identify the anti-tumor effects of baicalein on OS, three OS cells including 143 B, MG63 and U2OS cells were treated with various concentration of BA. The cell viability was measured and BA was found to significantly inhibit the cell proliferation in a concentration-dependent manner (Fig. 1A). The IC50 value of BA was 81.44, 131.8 and 53 μM for a 48-h treatment, respectively. We therefore selected the concentration of 80 μM for the further investigation. The results of colony formation indicated fewer and smaller colonies in BA-treated OS cells (Fig. 1B-C).

3.2. BA induced cell cycle arrest and apoptosis in OS cells

We next investigated the reason why BA inhibited cell viability in OS cells. The three OS cells were treated with 80 μM BA for 48 h, and subjected for cell cycle analyses. It was showed that the percentage of cells in G1-phase was increased in MG63 and U2OS cells; and higher percentage of cells in S-phase was in 143 B cells (Fig. 2A). Moreover, BA induced more apoptotic cells in the three OS cells (Fig. 2B). These results indicated that BA induced cell cycle arrest and apoptosis in OS cells.

3.3. BA suppressed metastasis of OS cells

We next investigated the suppressive effects of BA on invasion and metastasis in OS cells. As shown in Fig. 3A–B, the potential of cellular migration was inhibited by BA through the wound healing examination.

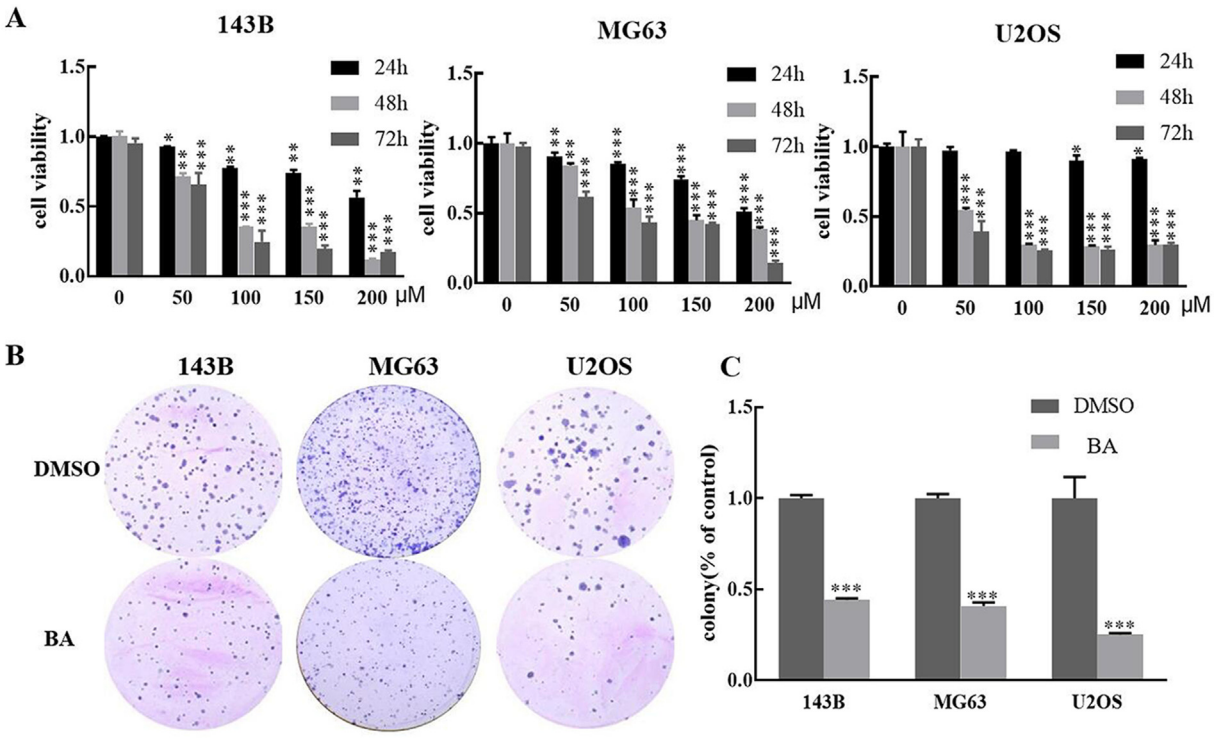

Figure 1. BA significantly suppressed cell viability in OS cells. A, 143 B, MG63 and U2OS cells were treated with serial concentrations of BA, and the effects of BA on cell proliferation were measured by Cell Counting Kit-8 examinations at 24, 48 and 72 h. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* 0. B–C, the three OS cells were treated with 80 μM BA for 14 days and the colony formation was examined. *, $P < 0.05$; **, $P < 0.01$; *vs* DMSO.

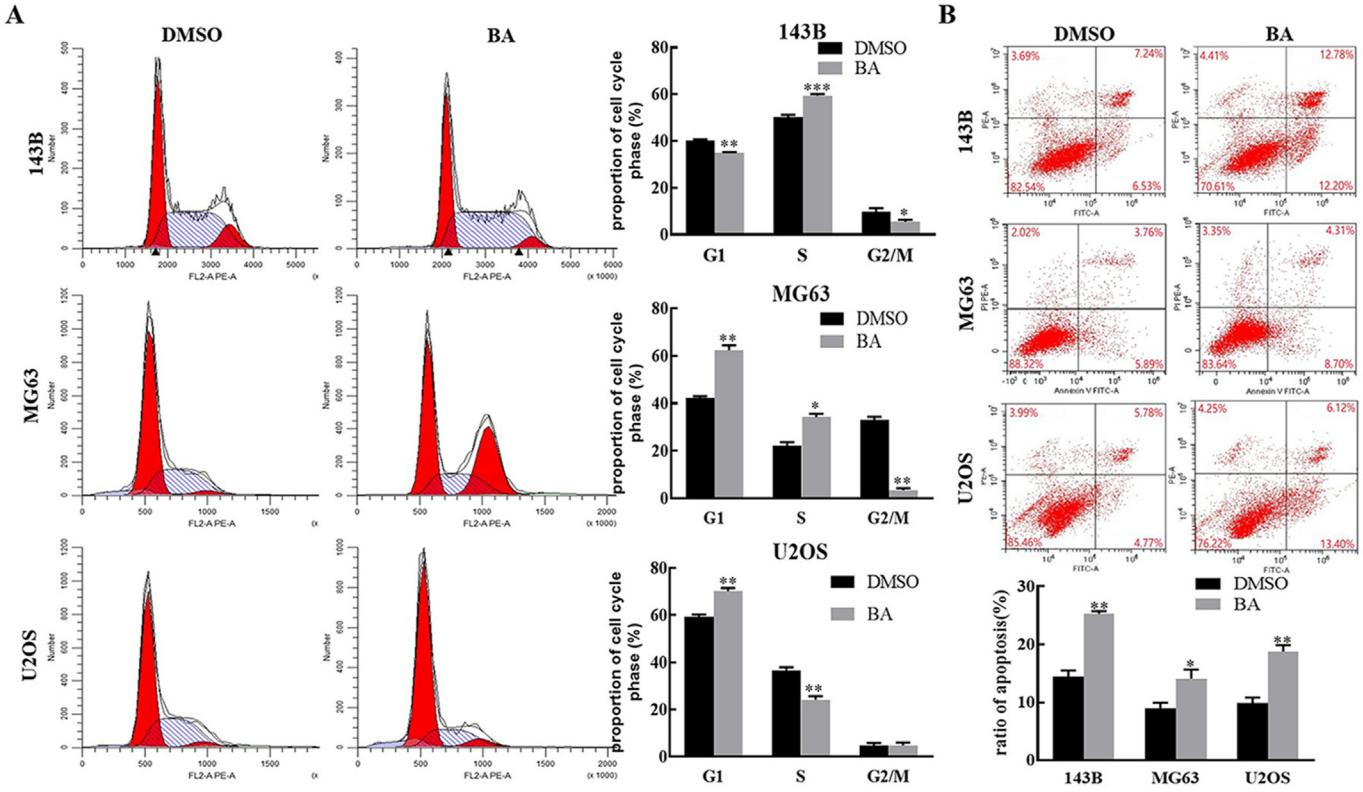

Figure 2. BA induced cell cycle arrest and apoptosis in OS cells. A, 143 B, MG63 and U2OS cells were treated with 80 μM BA for 48 h, then harvested and subjected for cell cycles analyses. B, After treatment, the apoptotic cells were examined by Annexin V-FITC and PI double staining. *, $P < 0.05$; **, $P < 0.01$; *vs* DMSO.

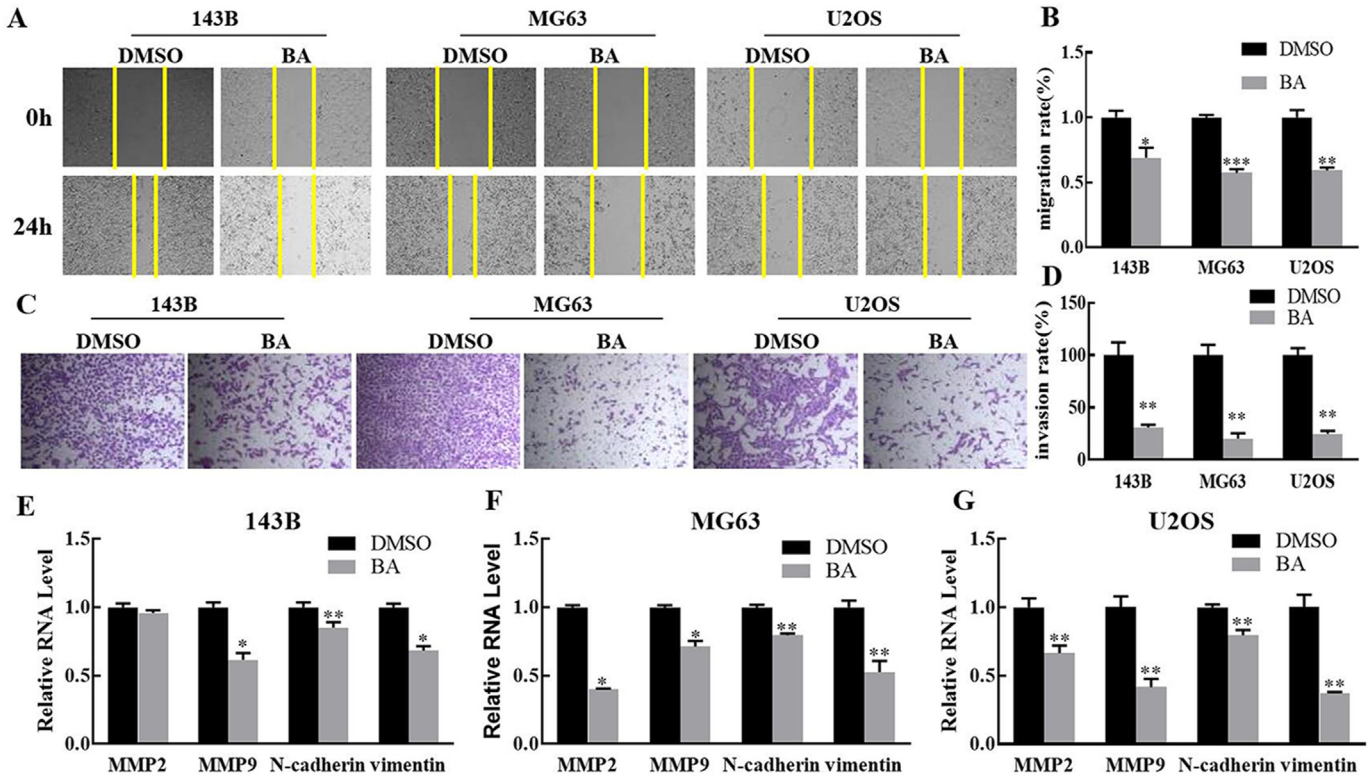

Figure 3. BA inhibited the invasion and metastasis of OS cells. A, The three OS cells were treated with 80 μM BA for 48 h, and wound-healing migration was assayed. B, The semi-quantitative examination of wound-healing areas in the three cells. C-D, transwell invasion of the BA-treated cell lines and the quantitative assays were examined. Data are presented as mean ± SD. E-G, The RNA expression levels of MMP2, MMP9, N-cadherin and vimentin were measured by qPCR examination in 143 B (E), MG63 (F) and U2OS (G) cells. *, $p < 0.05$; **, $p < 0.01$; ***, $P < 0.001$; *vs* DMSO.

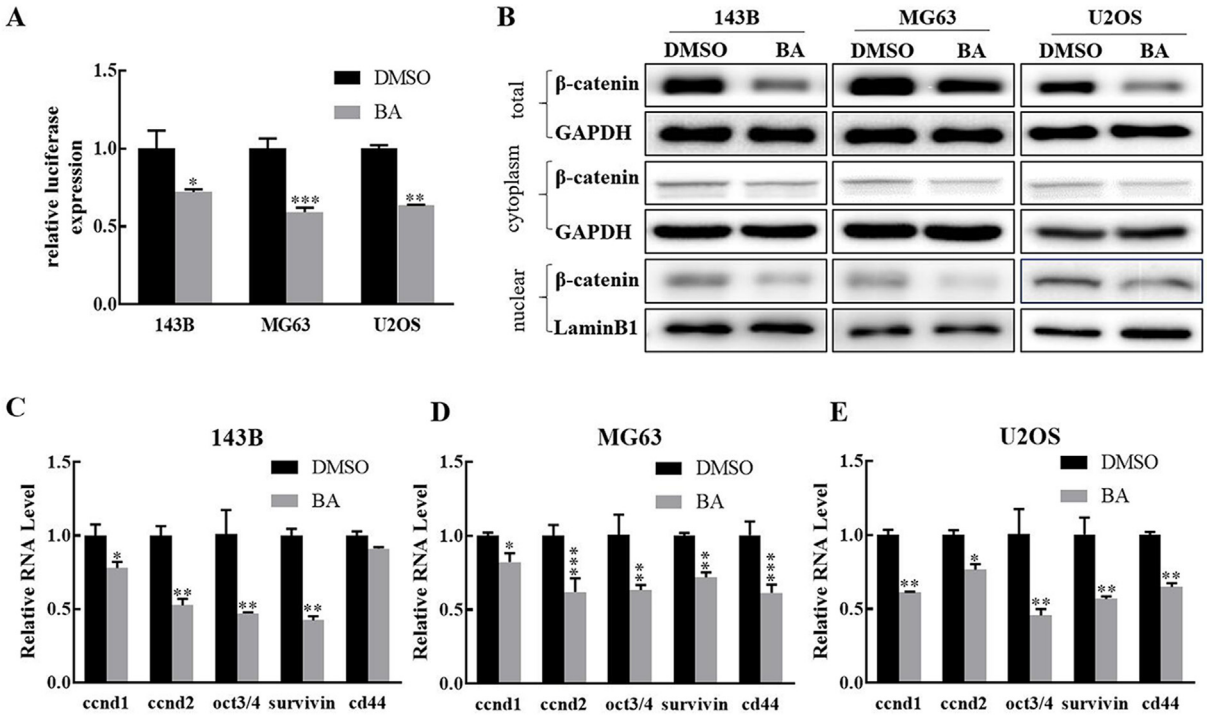

Figure 4. BA suppressed the Wnt/β-catenin signaling in OS cells. A, After transfected with TOPflash luciferase reporter, the three OS cells were treated with 80 μM BA for 48 h. The luciferase activities were measured. B, The total, cytoplasmic and intranuclear β-catenin were examined at protein level. Lamin B1 (nuclear expression) and GADPH (cytoplasmic expression) were used as the loading controls. C-E, The expression of several downstream targets of Wnt/β-catenin pathway were examined by qRT-PCR assays in the three OS cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* DMSO.

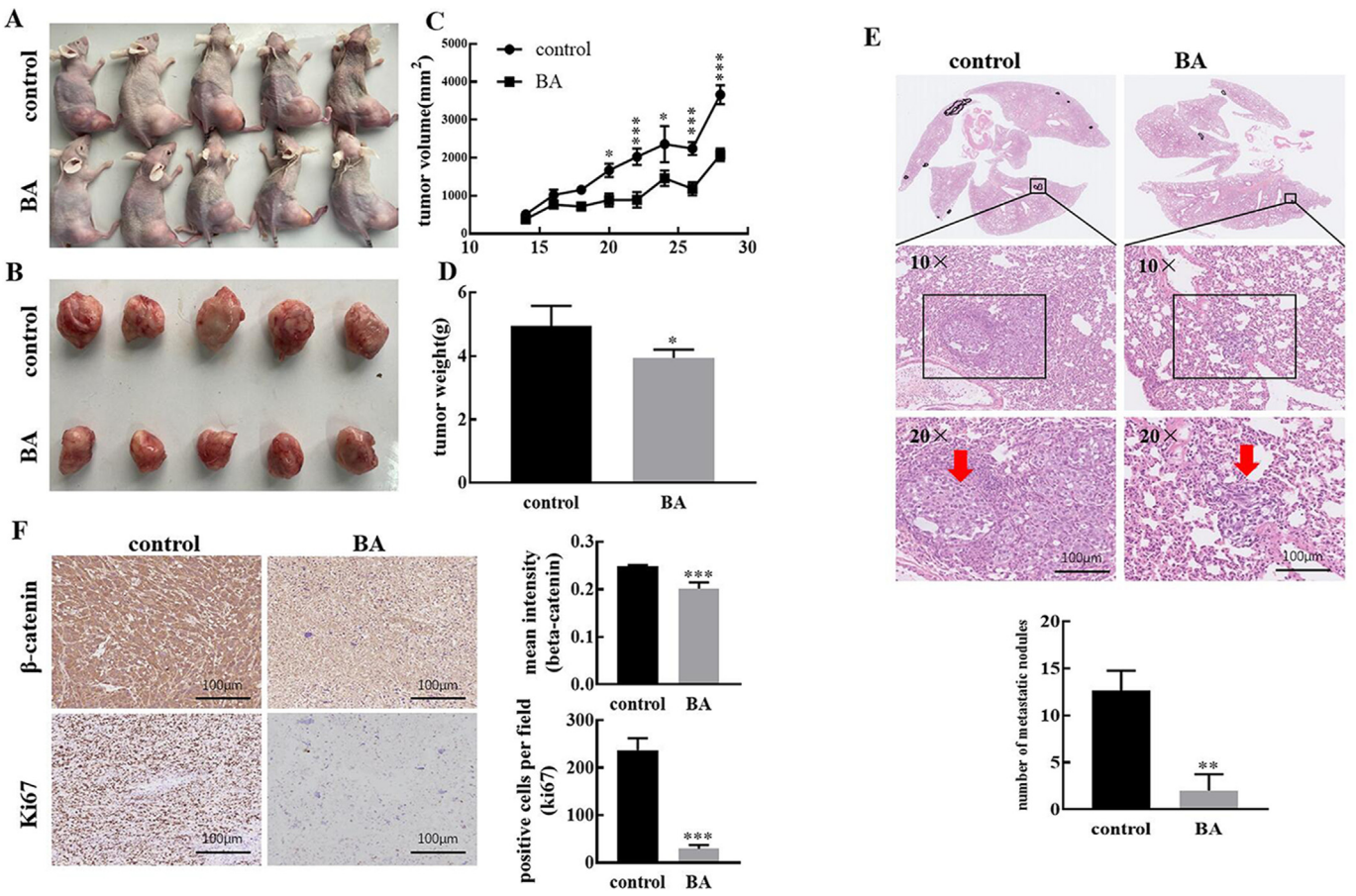

Figure 5. BA reduced the tumor growth and metastasis of OS cells *in vivo*. MG63 cells were injected into the medullary cavity of the right tibia of nude mice, and BA was administrated *i. p.* A-B, The images showed smaller tumors in BA treated groups when compared with saline groups. C, The growth curve of tumor volumes. D, The tumor weight. Each data represented the mean ± SEM of five mice. E, H&E staining for the lung tissue showed less numbers of metastatic nodules in the BA-treated mice. F, The immunofluorescence of Ki-67 and β-catenin stained sections followed by counterstaining with DAPI. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *vs* saline.

The further transwell assays exhibited less invaded cells in BA-treated cells than control groups (Fig. 3C–D), which was consistent with the wound healing results. We also monitored the expression of metastatic and epithelial mesenchymal transition (EMT) related genes such as MMP2, MMP9, N-cadherin and vimentin, and the results showed that their expression was significantly suppressed by BA in OS cells (Fig. 3E–G).

3.4. BA induced the inactivation of Wnt/β-catenin signaling in OS cells

As well known, Wnt/β-catenin signaling is an important cellular signal transduction pathway and it plays a critical role in the development of tumorigenesis [26,27]. We therefore wondered whether this signaling participated in the BA-mediated anti-OS activity. To validate our hypothesis, the luciferase reporter of Wnt signaling TOPflash, was transfected into OS cells, and it was showed that BA significantly suppressed the luciferase activities in all the OS cells (Fig. 4A). β-catenin, a key component of the Wnt signaling, interacts with transcription factors TCF/LEF and activates the downstream target genes of Wnt signaling [28]. Our results showed the total β-catenin expression was significantly reduced by BA in OS cells (Fig. 4B). The translocation of β-catenin from cytoplasm to nucleus stimulates Wnt signaling, and we found the expression of the cytoplasmic β-catenin remained unchangeable with BA treatment (Fig. 4B). We also observed that less nuclear β-catenin was enriched in the BA-treated HCC cells (Fig. 4B). Furthermore, several downstream target genes of Wnt/β-catenin signaling such as CD44, Oct3/4, CCND1, CCND2 and survivin were examined and the results showed that their expression was suppressed by BA in OS cells (Fig. 4C–E). All these results suggested that BA alleviated the canonical Wnt/β-catenin pathway *via* disrupting its translocation from cytoplasm to nucleus.

3.5. BA suppressed tumor growth and migration of OS cells in vivo

We next examined the *in vivo* function of BA in the tumorigenesis using an orthotopic intra-tibia tumor-bearing model. 1×10^6 MG63 cells were injected into the medullary cavity of the right tibia of nude mice, and BA (40 mg/kg) was administered intraperitoneally (*ip*) every day. Strikingly, the treated groups carried smaller burden when compared with control groups (Fig. 5A–B). We also observed a significant reduction in tumor growth (Fig. 5C) and weight (Fig. 5D) in BA-treated groups. The further H&E staining showed that BA administration significantly suppressed tumor growth *in vivo* (Fig. 5E). Ki67 has been considered as a significant cellular marker of proliferation, and a decreased expression was observed in tumor specimens derived from BA-treated groups by immunofluorescence staining (Fig. 5F). And we also found that the β-catenin expression was suppressed in treated groups as well (Fig. 5F).

3.6. BA suppressed cell viabilities and metastasis through promoting lncRNA-NEF expression in OS cells

Our previous study demonstrated that lncRNA-NEF mediated metastasis and epithelial to mesenchymal transition (EMT) in HCC cells [18]. In the present study, we monitored the expression of lncRNA-NEF in BA

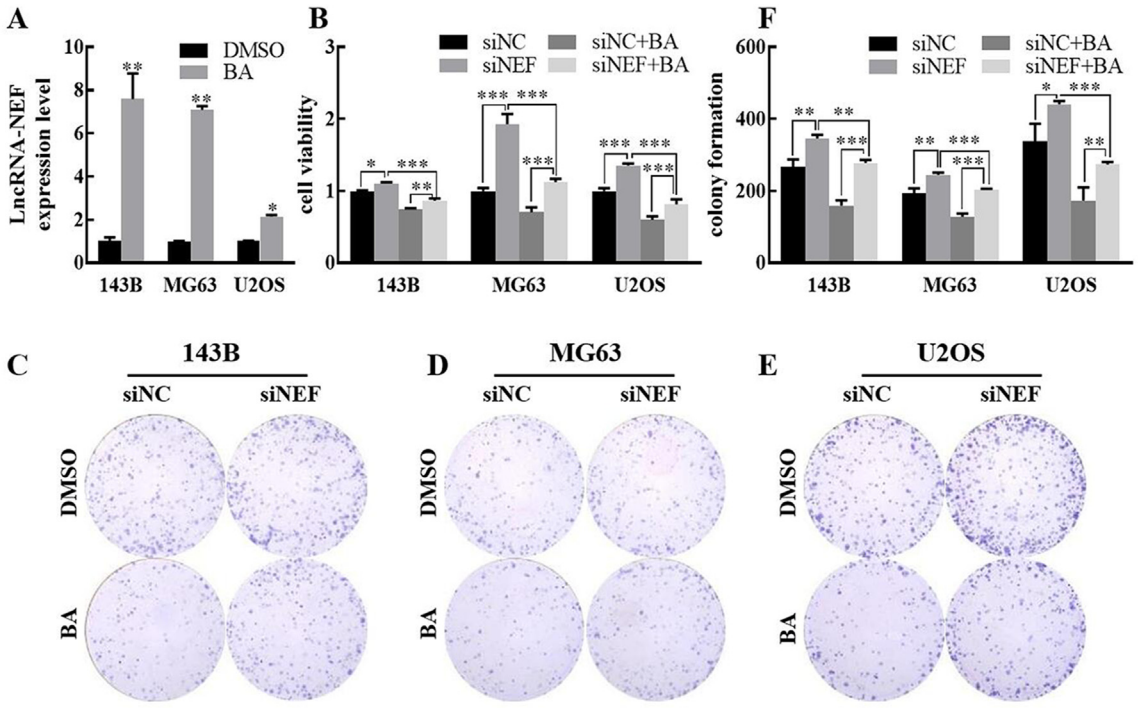

Figure 6. BA suppressed cell viability in OS cells *via* lncRNA-NEF up-regulation. A, The expression of lncRNA-NEF was obviously upregulated by BA in the three OS cells. *, P < 0.05; **, P < 0.01; *vs* DMSO. B–F, The cell viability (B) and colony formation (C–F) were examined in BA treated the siNEF transfected OS cells. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

treated OS cells. And the results showed that LncRNA-NEF were obviously up-regulated by BA (Fig. 6A). To further clarify whether lncRNA-NEF participates in BA-mediated anti-OS activity, the specific small interfering RNA for lncRNA-NEF (siNEF) was designed for the rescue study. Both cell viability (Fig. 6B) and colony formation (Fig. 6C–F) investigation showed that LncRNA-NEF silence partially attenuated the BA-induced inhibitory effects on OS cells. On the other hand, the further wound healing and transwell investigation showed that lncRNA-NEF knockdown alleviated the inhibitory effect of BA on metastasis potential (Supplementary Fig. S1, Fig. 7A–C). We also

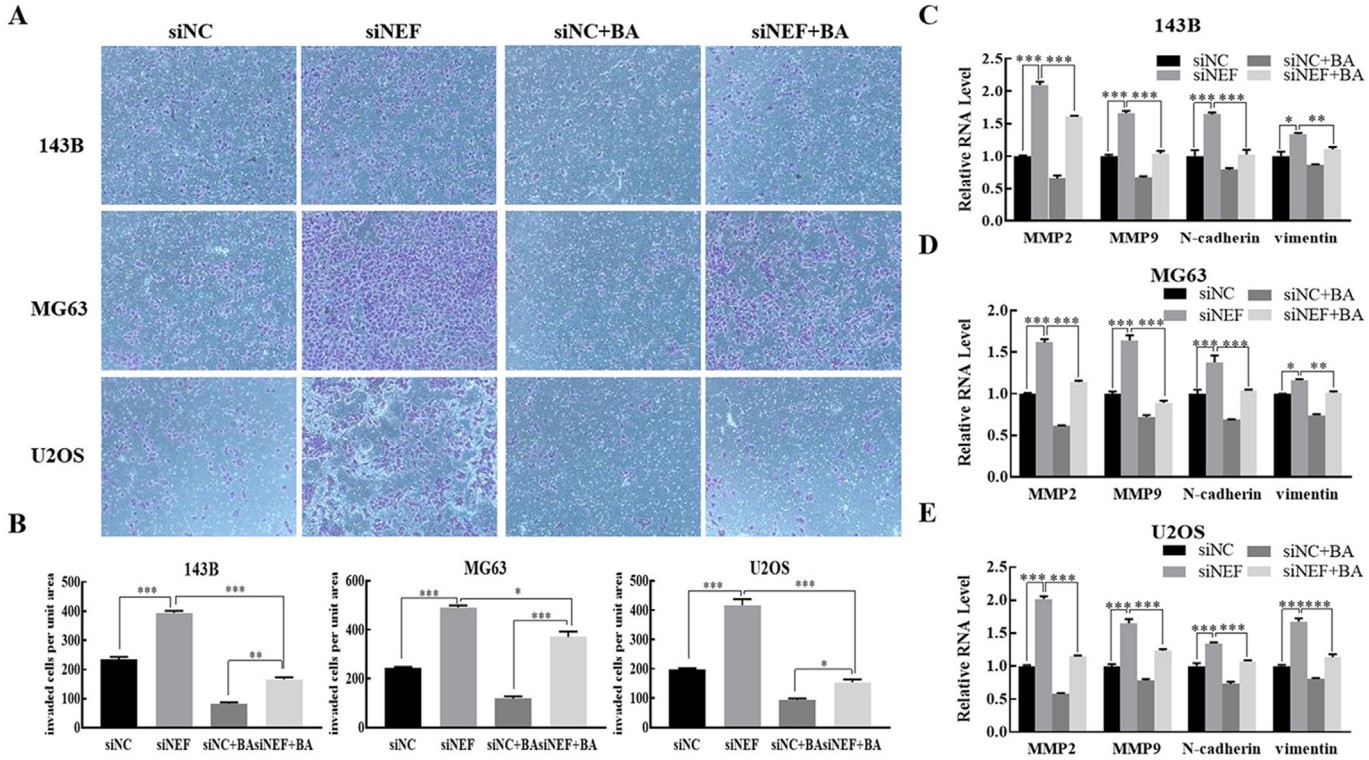

Figure 7. LncRNA-NEF knockdown partially attenuated the BA-induced inhibitory effects on metastasis in OS cells. A-B, transwell invasion of the BA-treated cell lines and the quantitative assays were examined. Data are presented as mean ± SD. C-E, The RNA expression levels of MMP2, MMP9, N-cadherin and vimentin were measured by qPCR examination in 143 B (C), MG63 (D) and U2OS (E) cells. *, $p < 0.05$; **, $p < 0.01$; ***, P < 0.001.

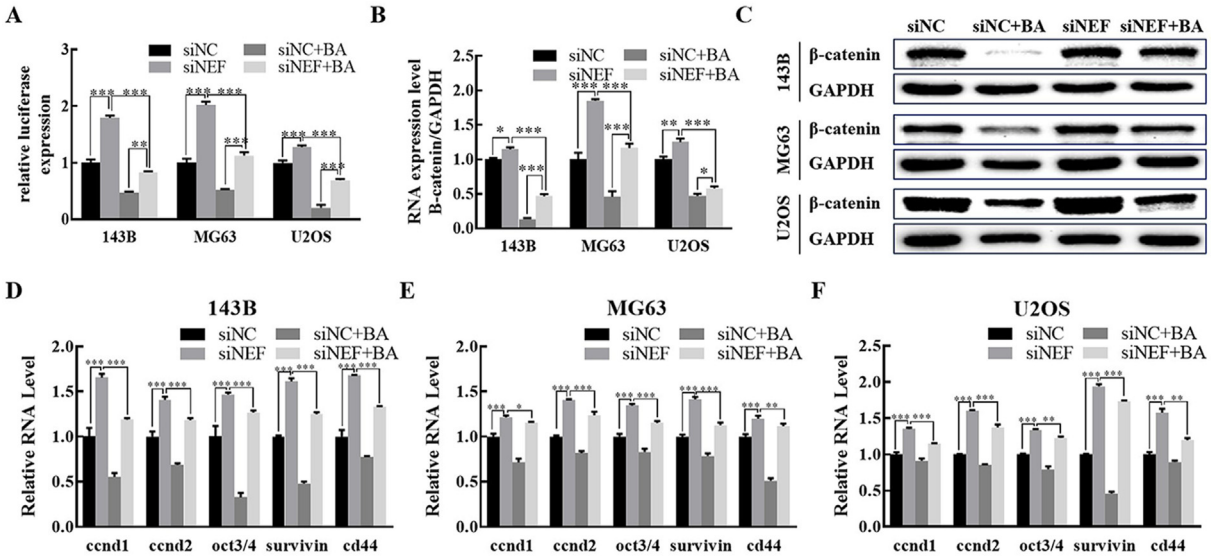

Figure 8. lncRNA-NEF knockdown partially reversed the inactivation of Wnt/β-catenin signaling induced by BA in OS cells. A, the three OS cells were cotransfected with siNEF and TOPflash luciferase reporter, then treated with 80 μM BA for 48 h. And the luciferase activities were measured. B, the RNA level of β-catenin was examined by qPCR examination after treatment with BA in lncRNA-NEF-silenced OS cells. C, the protein level of nuclear β-catenin was examined by Western blotting after treatment with BA in lncRNA-NEF-silenced OS cells. D-F, Several downstream target genes of Wnt/β-catenin signaling were examined by qPCR examination after BA treatment in siNEF transfected 143 B (D), MG (E) and U2Os (F) cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

evaluated RNA expression level of MMP2, MMP9, N-cadherin and vimentin, and it was showed that the BA-induced the down-regulation of these genes were significantly reversed by lncRNA-NEF knockdown in OS cells (Fig. 7C–E).

3.7. BA suppressed the tumor growth via the lncRNA-NEF driven Wnt/β-catenin signaling

Our previous study has demonstrated that lncRNA-NEF inhibited migration *via* regulating Wnt/β-catenin signaling in HCC. We therefore hypothesized that lncRNA-NEF mediated Wnt/β-catenin signaling might participate in the BA-induced anti-cancer activity. As shown in Fig. 8A, our results showed that lncRNA-NEF knockdown significantly abolished the suppressive effect on luciferase activity of TOPflash. Furthermore, the knockdown of lncRNA-NEF obviously compensated the inhibitory expression of total β-catenin (Fig. 8B–C) and several downstream target genes of Wnt/β-catenin signaling (Fig. 8D–F), thus partially cancelled the inactivation of Wnt/β-catenin signaling in BA-treated OS cells.

4. Discussion

Although OS has a low incidence worldwide, it is an important cause of cancer-related death in pediatric population which bring heavy burden to family and society [29]. Treatment of OS clinical practice remains unsatisfactory mainly due to a high risk of metastasis. Traditional Chinese medicine (TCM) provides a promising approach for OS management [30]. Compared with the traditional chemotherapy, natural active constituents extracted from Chinese herbs have less adverse reaction and better anti-tumor activities, which make them to be developed as potential, novel anti-cancer drugs for OS patients [31].

Baicalein (BA) is an important bioactive flavonoid extracted from Chinese herb *Scutellaria baicalensis*. Increasing evidence have demonstrated that BA has fascinating anti-cancer properties in various cancers such as breast cancer [32], colorectal cancer [33], lung cancer [34], cervical cancer [35], thyroid cancer [36], etc. BA was reported to inhibit the proliferation, induce apoptosis and autophagy, and suppress epithelial–mesenchymal transitions (EMT) and metastasis as well [37]. On the other hand, BA also serves as an adjuvant to improve the effect of chemotherapy on cancer progression [38]. BA increased the cisplatin sensitivity of lung adenocarcinoma cells [39]. In the present study, we systematically investigated the anticancer effects of BA on OS *in vitro* and *in vivo*. By cell viability and metastasis assays, BA was found to suppress cell viability *via* inducing cell cycle arrest and apoptosis as well as migration and invasion. The further orthotopic intra-tibia tumor-bearing model confirmed the anti-cancer activity of BA *in vivo*. Recent evidence also demonstrated that BA inhibited cell development, metastasis and EMT and induced apoptosis in OS cells [40], which was consistent with our results. However, the underlying mechanism of BA remains spare and elusive. Our study firstly revealed that lncRNA driven Wnt/β-catenin signaling participated in the process of BA-suppressed tumor growth.

Wnt/β-catenin signaling is one of the key cascades regulating development and differentiation, and plays an important role in tumorigenesis [41,42]. The role of Wnt signaling in carcinogenesis has most prominently been described in various cancers [43]. Aberrant activation of Wnt signaling has been implicated in human osteosarcoma, which may provide a genetic vulnerability that can be targeted in OS. Therefore, inhibiting the Wnt/β-catenin signaling may be a promising strategy for OS therapeutics. Recent studies have revealed that BA suppressed the Wnt/β-catenin signaling in multiple cancers including OS [44,45]. In the present study, BA was found to induce the inactivation of Wnt/β-catenin signaling through the examination of the luciferase activity of TOPflash reporter, β-catenin expression and downstream genes of this signaling. However, how BA suppressed the Wnt/β-catenin signaling remains obscure.

LncRNA represents a new member of heterogeneous noncoding RNAs (ncRNAs), and its emerge provides a bridge between TCM and their function [46]. As a kind of critical regulator, lncRNAs have been demonstrated to mediate signal transduction in various biological activities and diseases. Several lncRNAs was reported to mediate the Wnt/β-catenin signaling in tumorigenesis [47]. For example, lncRNA CCAT2 promoted breast tumor progression by stimulating β-catenin expression, thereby activating the Wnt signaling [48]. Another lncRNA HOTAIR was found to suppress the expression of Wnt-1, thereby inhibiting the activation of Wnt/β-catenin signaling and promoting the apoptosis of synovial cells [49]. Our previous study showed that lncRNA-NEF induced the inactivation of Wnt/β-catenin signaling to antagonize cancer metastasis in HCC [18]. In this study, lncRNA-NEF was significantly up-regulated by BA in OS cells, indicating that this lncRNA

may involve in BA-induced anti-cancer activity. To further confirm the involvement of lncRNA-NEF in this anti-cancer activity, siNEF was designed to perform the rescue study. Our results showed that lncRNA-NEF knockdown partially alleviated the BA-induced inhibitory effects on cell growth, metastasis and invasion in OS cells; and successfully reversed the inactivation of Wnt/β-catenin signaling as well. These data indicate that lncRNA-NEF driven Wnt/β-catenin signaling regulatory axis participates in the BA-mediated anti-cancer activity.

In summary, our data demonstrated that BA played anti-tumor roles in OS through an epigenetic regulatory pattern in which lncRNA-NEF expression was suppressed as well as Wnt/β-catenin signaling, thus resulted in the inhibition of tumor growth and metastasis. The findings provide a novel mechanism of the BA-mediated tumor suppression and suggest BA may be developed as a promising candidate for OS patients.

Declaration of competing interest

All authors declared no potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2021.12.001.

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