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ESPN activates ZEB1-mediated EMT through the PI3K/AKT/mTOR axis to promote osteosarcoma metastasis

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Abstract

Background Osteosarcoma (OS) is a primary bone malignancy characterized by early metastasis and generally poor prognosis. ESPN is highly expressed and plays an important role in regulating the aggressive phenotypes of several cancer cell types. However, little is known about the molecular mechanisms underlying ESPN-mediated migration and invasion in OS cells.

Methods In this study, we first analyzed the survival of osteosarcoma patients using Kaplan-Meier analysis to assess the prognostic relevance of ESPN. To further evaluate its clinical significance, we performed immunohistochemical analysis on osteosarcoma tissue samples and benign osteochondroma (OC) tissues. The biological function of ESPN in osteosarcoma was confirmed by a series of experiments conducted both in vitro and in vivo. Additionally, we explored the underlying molecular mechanisms through Western blotting, co-immunoprecipitation, immunofluorescence, and PCR, revealing key downstream signaling pathways.

Results In this study, we demonstrate that ESPN, acting as an oncogene, is highly expressed in OS cell lines and tissues, promoting OS cell proliferation and metastasis. Mechanistically, ESPN promoted the phosphorylation of PI3K by direct interaction with it and active the AKT/mTOR pathway, which enhanced the expression of the transcription factor ZEB1 and initiating the epithelial-mesenchymal transition (EMT) cascade. Furthermore, we validated that mTOR-mediated activation of p70 ribosomal protein S6 kinase (p70S6K) promotes the translation of ZEB1, thereby enhancing the growth and motility of OS cells.

Conclusions Our findings reveal a previously unrecognized function of ESPN in OS, closely linked with EMT and cancer metastasis progression. Targeting ESPN may represent a potential therapeutic approach for patients with OS.

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Keywords ESPN, Epithelial-mesenchymal transition, mTOR, Osteosarcoma, Metastasis

Introduction

Osteosarcoma (OS) is the most common malignant primary bone tumor [1]. It primarily affects children, adolescents, and young adults, with a median age of 16 years, and poses a significant risk for mortality [2, 3]. Highly aggressive OS can quickly invade surrounding tissues, leading to distant metastasis, with the lungs being the most common site of metastasis [4]. Approximately 20% of OS patients present with metastasis at the time of diagnosis [5]. The current standard treatment for localized osteosarcoma involves a neoadjuvant chemotherapy-surgery-consolidation chemotherapy approach, which has achieved significant clinical success [6]. However, for patients with metastasis at diagnosis or recurrence, the prognosis remains poor, with a 5-year survival rate of only 20–30% [7]. Given the limitations of these conventional treatment regimens, there is an urgent need to develop new therapeutic strategies to improve outcomes for osteosarcoma patients.

ESPN belongs to the actin-binding protein family and is localized in the microvilli of various sensory cells [8]. ESPN can regulate F-actin by controlling intracellular cytoskeletal remodeling, thereby affecting F-actin organization and signaling [9]. Dynamic remodeling of the actin cytoskeleton is necessary for cancer invasion [10]. The continuous polymerization and depolymerization of actin is a key factor in the EMT process [11]. In Huang's study reducing CAPZA1 expression inhibits EMT in HCC cells by regulating actin cytoskeleton remodeling, thereby reducing cell metastatic ability [12]. Likewise, ESPN has been proposed to participate in the metastatic process of cancer, the migration of esophageal squamous carcinoma cancer cells can be effectively inhibited by reducing the expression of ESPN [13]. MiR-612 suppresses the aggressive phenotype of melanoma cells by down-regulating the ESPN [14]. Collectively, ESPN may play a crucial role in controlling cancer progression. However, to our knowledge, the expression of ESPN in OS and whether it is involved in the development of osteosarcoma remains elusive.

In the present investigation, we found that ESPN expression is increased in OS and is positively correlated with clinical prognosis. Moreover, for the first time, we demonstrated that ESPN directly interacts with PI3K to activate the AKT/mTOR/S6K/S6 signaling pathway, thereby regulating ZEB1 expression and promoting EMT progression and metastasis in OS.

Materials and methods

Cells culture and reagents

Human osteoblast hFOB1.19 cells were obtained from the Cells Bank of the Chinese Academy of Sciences (Shanghai, China). Human osteosarcoma MG63, U-2OS, MNNG-HOS and 143B cells lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA). All cell lines were maintained according to standard protocols. Briefly, U-2OS, MNNG-HOS, MG63 and 143B cells were maintained at 37 °C in a 5% CO_2 environment; hFOB1.19 cells were maintained at 34.5 °C in a 5% CO_2 environment. Antibodies against the following targets were used. ESPN (A15908 ABclonal), E-cadherin (A20798 ABclonal), N-cadherin (A19083 ABclonal), Vimentin (A11952 ABclonal), AKT (10176-2-AP Proteintech), p-AKT (66444-1-lg Proteintech), PI3K (A4992 ABclonal), p-PI3K (AP0854 ABclonal), S6K1 (ab203558 abcam), p-S6K1 (ab59208 abcam), S6 (A11874 ABclonal), p-S6 (ab80158 abcam), GAPDH (SB-AB0037 share bio), Snail (#3879 Cells Signaling Technology), ZEB1 (#70512 Cells Signaling Technology), p-mTOR (#2971 Cells Signaling Technology), mTOR (#2983 Cells Signaling Technology), Slug (A1057 ABclonal), Twist (A3237 ABclonal), and Notch1(A19090 ABclonal), Notch2(V1697 Abways), Notch3(A13522, ABclonal), Hes1(A0925, ABclonal), Smad2(CY5090, Abways), p-Smad2(CY5857, Abways), Smad3(CY5090, Abways), p-Smad3(CY6485, Abways), TCF4(CY8810, Abways), β-cantenin(#9562, Cells Signaling Technology), Cyclin D(CY2970, Abways), P65(A19653, ABclonal), p-P65(AP1294, ABclonal), IκBα(A24909, ABclonal), p- IκBα(AP0707, ABclonal), IgG(ShareBio, SB-A00002), HA(ab9110, abcam) and Myc(ab9106, abcam). Pharmacological agonist or inhibitors were added to cells for the time and at the concentration, Rapamycin (HY-10219; MCE), 3BDO (HY-U00434; MCE), PF-4708671(HY-15773; MCE).

RNA extraction and quantitative real-time PCR (qRT–PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen, USA), and cDNA was synthesized using the Quantscript RT kit (Vazyme, China). In qRT−PCR, relative gene expression was measured by a SYBR RT PCR kit (Biomake, USA). qRT-PCR experiments were conducted as previously described [15].

Transfection assay

Stable ESPN knockout or ZEB1 knockout OS cells (MNNG-HOS and 143B) were constructed. The sh-ESPN sequences were as follows:

sh-1: 5'-GGUGAACUGGCUCUUGCAUT, AUGCAAG AGCCAGUUCACCTT-3'.

sh-2: 5'AUGCAAGAGCCAGUUCACCTT, AUGCAAG AGCCAGUUCACCTT-3'.

The ZEB1 shRNA sequence was as follows: 5'-CGGCG CAATAACGTTACAAAT-3'.

ESPN shRNA plasmid, ZEB1shRNA plasmid and its negative control were purchased from Gene Pharma. The plasmid sets containing ESPN overexpression, ZEB1 overexpression, HA-ESPN, MYC-PI3K, and the negative control plasmids were purchased from OBiO Technologies. We packaged these plasmids into virus particles using HEK 293 T cells and determined the viral titers. To obtain stable knockdown cell lines and overexpressing cell lines, target cells were seeded on six-well plates and coinfected with 1×10^8 lentivirus-transducing units and polybrene (Sigma−Aldrich, TR-1003). After 72 h, the infected cells were screened with 2.5 μg/mL puromycin. Finally, the interference efficiency was verified by qRT-PCR and Western blot.

Western blot

Cells were first lysed on ice for 10 min to extract whole-cells proteins, and then protein concentration was measured using the BCA Protein Quantification Kit. Equal amounts of protein were then electrophoresed on 8% and 10% sodium dodecyl sulfate polyacrylamide gels. After the protein was electrotransferred to the membrane and blocked, the membrane was blocked with 5% skim milk for 1 h at room temperature, incubated with primary antibody overnight at 4 °C and washed three times with TBST. The membranes were incubated with the secondary antibody for 1 h at room temperature. Finally, the membrane was detected using Share-Bio's enhanced chemiluminescence reagent in combination with Western electrochemiluminescence (ECL) substrate.

Migration and invasion assays

The transwell assay was used to assess cells migration and invasion, and the transwell chamber had 24 wells with or without Matrigel substrate (Corning, USA). In brief, 2×10^4 MNNG-HOS, 143B, and MG63 cells were cultured in the upper chamber with 200 μl of serum-free medium. Then, 500 μl of medium containing 10% fetal bovine serum was added to the lower chamber. After 48 h of incubation, cells that had migrated or invaded the membrane were fixed with 4% formaldehyde and stained with 0.1% crystal violet, while the cells that did not migrate or invade the upper chamber were wiped off. Finally, cells were randomly photographed and counted under a microscope.

Wound healing assay

Cells migration was detected using the trauma repair assay. Briefly, when the fusion of 143B, MNNG-HOS, and MG63 cells in a 6-well plate was approximately 80-90%, a wound was formed by scraping a crosshair across the fused cells layer with a 200 μl pipette tip. Rinse with PBS to remove floating cells before adding serum-free medium and repeat three times. Images of the scratch width at 0 h and 24 h were captured using a computerized microimaging system, and migration distance was calculated and compared by image analysis using ImageJ software.

Colony formation assay and CCK-8 assay

The CCK-8 and colony formation assays were performed as described previously [16].

Tail vein injection

To determine the role of ESPN in osteosarcoma metastasis, osteosarcoma cells were injected into the tail vein of 6-week-old male thymus-less BALB/c nude mice to observe differences in lung metastasis between groups. Mice were randomly divided into a negative control (sh-CON) group, a group with stable knockout of ESPN (sh-1), and a group with a knockout ESPN and concurrent overexpression of ZEB1 (sh-1 + ZEB1). Each mouse was injected with 2×10^6 143B cells. A control group (Vector), an overexpression of ESPN (ESPN) group, and an overexpression of ESPN with knockdown of ZEB1 (ESPN + sh-ZEB1) group were also established. Each mouse was injected with 2×10^6 MG63 cells and transfected accordingly. The mice were euthanized 4 weeks after injection, and lung tissues were obtained from the mice, followed by paraffin embedding and analysis. All animal experiments were approved by the Ethics Committee.

Immunohistochemistry (IHC)

Microarray contained tissues from 70 OS patients, another microarray contained tissues from 24 OS patients (Alina Biotech, China); IHC was performed as previously described [17]. The antibodies used were as follows: ESPN (1/100, A15908, ABclonal), ZEB1(1/400, #70512, Cells Signaling Technology), p-S6K (1/100, ab308331, Abcam). The final IHC score was calculated by multiplying the intensity score by the staining proportion score, as described previously [16]. Two experienced pathologists independently assessed the samples in a blinded manner and recorded the scores.

Molecular docking analysis

The HDOCK web server represents an integrated platform that combines molecular docking and template-based modeling, enabling the prediction of protein-protein interaction (PPI) models through rigid-body docking of macromolecules [18]. In this study, we employed this platform to predict the interaction model between human PI3K (4JPS.pdb) and mouse ESPN (5ET1.pdb), the latter exhibiting high structural

similarity to human ESPN. This approach provides structural insights into the potential interaction mechanism between these two proteins at the molecular level.

Co-immunoprecipitation (Co-IP) assay

MNNG-HOS and 143B proteins were extracted through total protein extraction buffer (Beyotime, China). Protein A/G sepharose (Santa Cruz Biotechnology) was pre-incubated with anti-HA or anti-MYC antibodies for 30–60 min on a spinning wheel at 4 °C with two washes. The bead-antibody complexes were then suspended with protein lysate. All Co-IP was performed overnight on a spinning wheel at 4 °C. The beads were washed 3 times with extraction buffer, and were collected by centrifugation at 3000 g. The immunoprecipitates were subjected to western blot.

Immunofluorescence analysis

Slides with treated cells were added to 24-well plates. The slides were fixed with 4% paraformaldehyde for 20 min when 60% of the cells were fused. The cells were permeabilized with 0.1% Triton X-100 (Sigma, USA) for 5 min. The cells sections were blocked with 5% bovine serum albumin for 1 h and then incubated with Vimentin (A11952 1/100; ABclonal), N-cadherin (A19083 1/100; ABclonal), and E-cadherin (A20798 1/100; ABclonal) antibodies at 4 °C overnight. The next day, the cells were incubated with DyLight 488-labeled sheep anti-rabbit antibody (H + L) or DyLight 594-labeled sheep anti-rabbit antibody (H + L) for 2 h in the dark and then stained with 4′,6-Diamidino-2-phenylindole (Beyotime Biotech, China) for 5 min. Finally, the cells slides were sealed with antifade mounting medium and stored at 4 °C in a dark environment, and immunofluorescence images were acquired using a fluorescence microscope.

Statistical analysis

Data was analyzed using GraphPad Prism 8.0 and SPSS 26.0. All experimental data are expressed as the mean ± standard deviation for all experimental data. All experiments were repeated three times. Statistical significance was achieved when the p value was less than 0.05.

Results

ESPN is upregulated in OS

Given the important role of ESPN in malignant tumors, we aim to analyze the prognostic value of ESPN in osteosarcoma patients using the R2 online database. Kaplan-Meier analysis showed that osteosarcoma patients with high ESPN expression have lower overall survival and metastasis-free survival rates (Fig. 1A). IHC results also indicated that ESPN positive expression rate was higher in OS tissues compared to benign osteochondroma (OC) tissues (Fig. 1B, C). High ESPN expression was associated with advanced pathological stages in patients (Fig. 1D). Additionally, we detected ESPN expression in a human osteoblast cell line (hFOB1.19) and human OS cell lines (MNNG-HOS, U-2OS, 143B, MG63). ESPN mRNA expression was significantly increased in OS cells compared to hFOB1.19 cells (Fig. 1E). Similar results were found at the protein level (Fig. 1F). Therefore, our data shows that ESPN is elevated in OS and is positively correlated with poor patient prognosis.

ESPN promotes OS cells migration, invasion and viability

To determine the role of ESPN in OS progression, we stably knocked down ESPN in 143B and MNNG-HOS cells using lentivirus. The efficiency of the knockdown was evaluated by qRT-PCR and Western blot (Fig. 2A and Supplement Fig. 1A). Scratch assay demonstrated that ESPN knockdown significantly slowed the migration of OS cells (Fig. 2B and Supplement Fig. 1B). Furthermore, transwell and invasion assays demonstrated that silencing ESPN significantly inhibited the motility of OS cells (Fig. 2C and Supplement Fig. 1C, D). We also performed CCK-8 and colony formation assays to assess the impact of ESPN knockdown on OS cell proliferation (Fig. 2D, E and Supplement Fig. 1E). When we overexpressed ESPN in MG63 cells, which have low endogenous ESPN levels (Fig. 2F), increased ESPN expression enhanced the motility and viability of OS cells (Fig. 2G-K and Supplement Fig. 1F, G). These results suggest that ESPN promotes the proliferation and motility of OS cells.

ESPN accelerates EMT in OS cells

EMT is involved in the metastasis and invasion processes of OS [19]. To explore whether the intrinsic mechanism by which ESPN promotes cell invasion and metastasis through EMT. We examined the expression of EMT-related markers in 143B, MNNG-HOS, and MG63 cells. Upon ESPN knockdown, the expression of mesenchymal markers Vimentin and N-cadherin was reduced, while the expression of the epithelial marker E-cadherin was increased (Fig. 3A). Conversely, ESPN overexpression exhibited the opposite effect (Fig. 3B). The immunofluorescence (IF) assay results in 143B and MNNG-HOS cells showed that the IF intensities of N-cadherin and Vimentin were reduced in ESPN-depleted cells, while the intensity of E-cadherin was increased (Fig. 3C, D, F). In addition, we observed an EMT-promoting effect in MG63 cells overexpressing ESPN (Fig. 3E, G). These results indicate that ESPN plays a crucial role in promoting EMT in OS.

ESPN enhances EMT by promoting ZEB1 expression

To elucidate the mechanism by which ESPN promotes EMT in OS, we examined the expression of EMT-related transcription factors via qRT-PCR and Western blot.

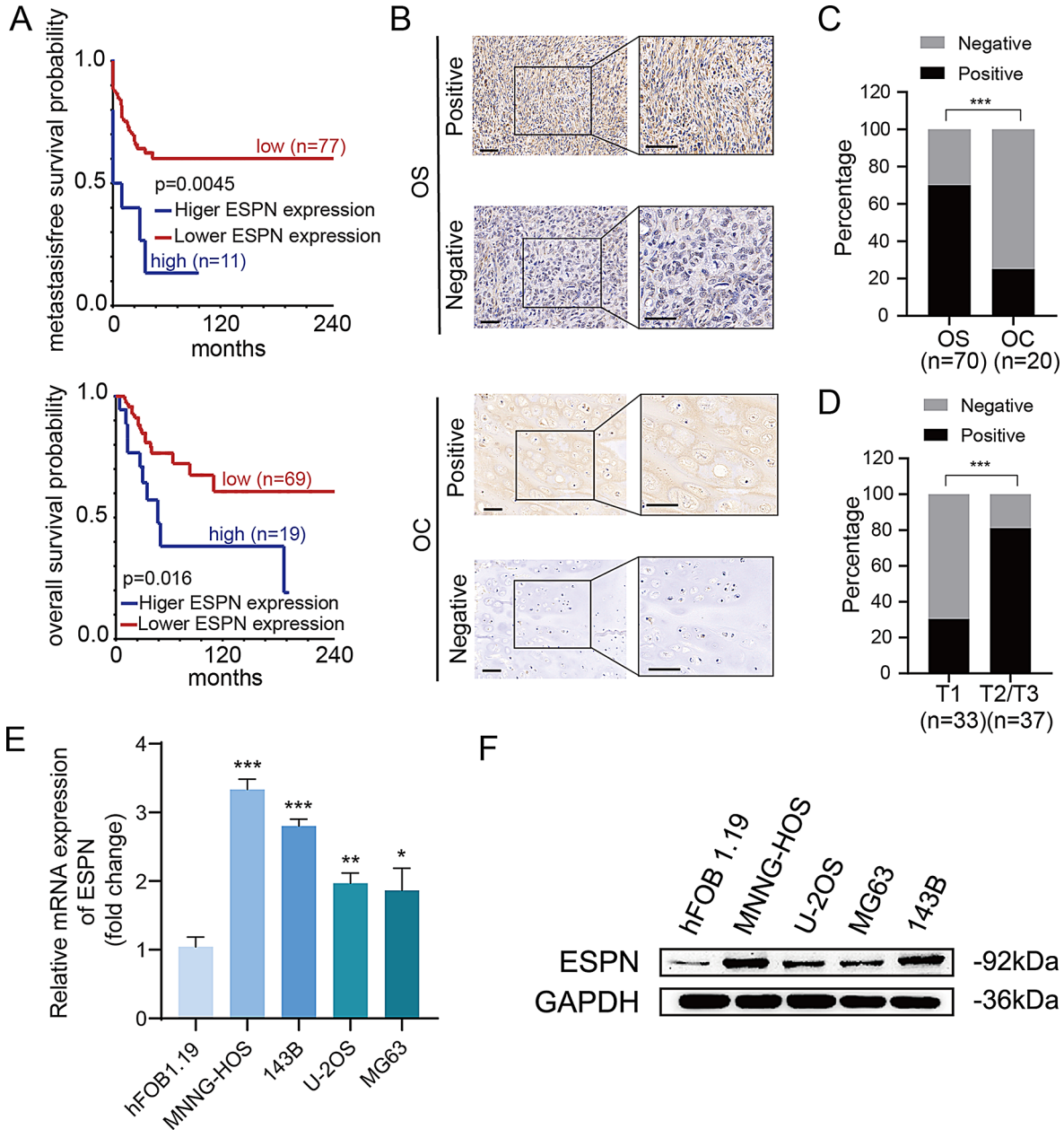

Fig. 1 Elevated ESPN expression in OS (**A**) Based on the Human Osteosarcoma Gene Expression Database (https://hgserver1.amc.nl/cgi-bin/R2/main.cgi), Kaplan–Meier analysis of the overall survival in 88-patient OS group by ESPN expression and metastasis status. (**B**) Immunohistochemical methods (IHC) were applied to detect ESPN expression in OS and osteochondroma (OC) tissues. (**C**, **D**) The results were statistically analyzed according to the expression of ESPN in OS and OC. (**E**) Quantitative polymerase chain reaction was used to detect the expression of ESPN in normal osteoblast cells lines (hFOB1.19) and OS cells lines (U-2OS, MNNG-HOS, MG63 and 143B). (**F**) Western blot for ESPN expression in a normal osteoblast cells line (hFOB1.19) and OS cells lines (U-2OS, MNNG-HOS, MG63 and 143B). Values are expressed as mean ± standard deviation, scale bar = 100 μm, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

Knockdown of ESPN had no effect on the mRNA levels of ZEB1, Snail, Slug, and Twist (Supplement Fig. 2A). However, we observed that ESPN knockdown significantly suppressed ZEB1 protein expression, and elevated ZEB1 levels were strongly associated with poor clinical prognosis in osteosarcoma patients (Fig. 4A and Supplement Fig. 2B, C). Next, we overexpressed ZEB1 in ESPN-deficient 143B and MNNG-HOS cells. Western blot analysis indicated that ZEB1 overexpression partially restored the expression of mesenchymal markers while simultaneously reducing the expression of epithelial markers (Fig. 4B). Moreover, the excessive expression of ZEB1 reversed the decreased OS cellular metastasis and invasive capacity caused by ESPN deficiency (Fig. 4C, D and Supplement Fig. 2D-F). Similarly, when ESPN overexpression merely promoted ZEB1 protein

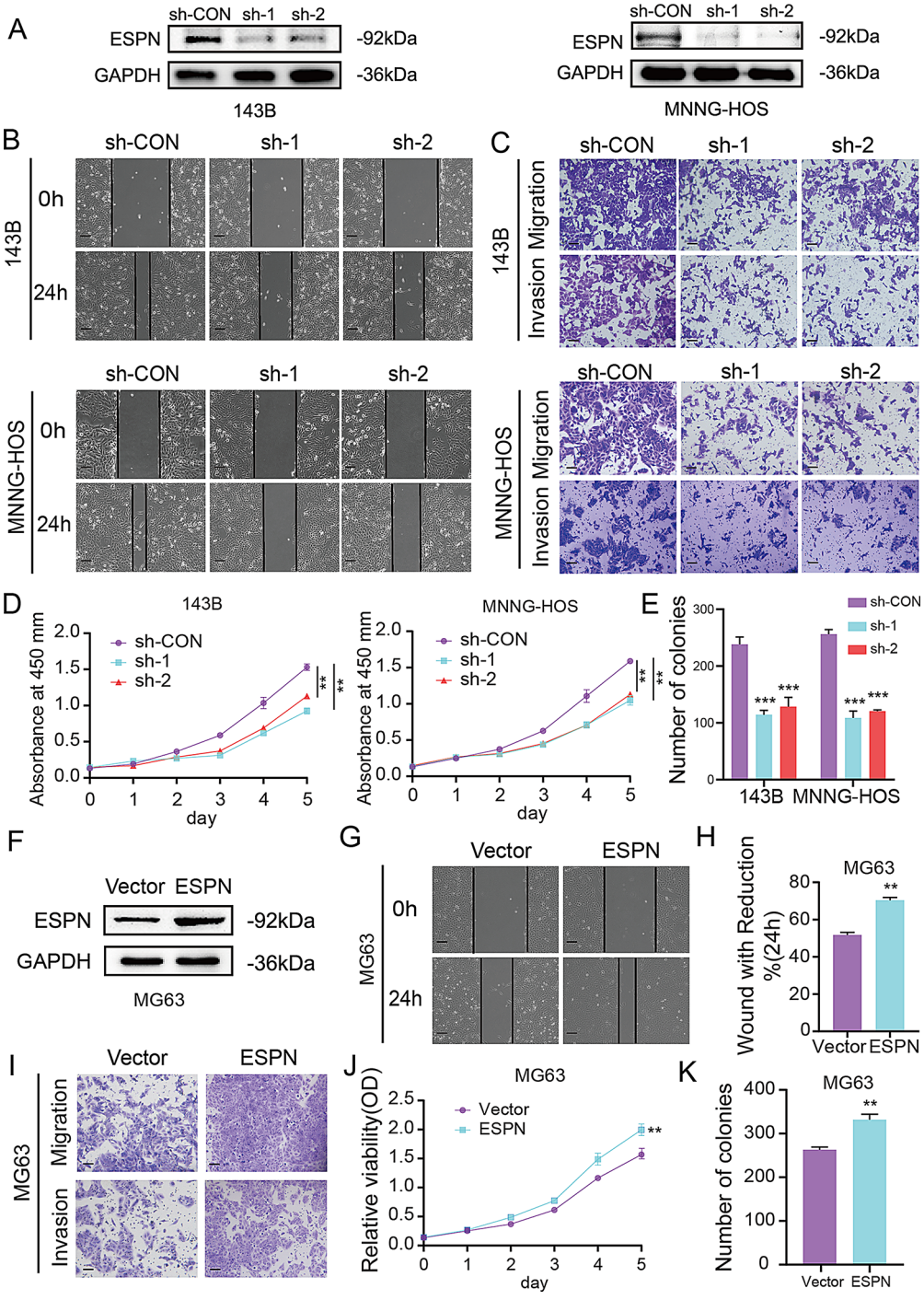

Fig. 2 ESPN promotes OS progression in vitro (**A**) Knockdown efficiency of ESPN in OS cells (MNNG-HOS and 143B) was detected by Western blot. (**B**) Scratch and (**C**) Transwell assays for cells migration and invasive ability. These data represent three independent experiments. (**D**) The results of CCK-8 experiments showed that ESPN knockdown inhibited the proliferative ability of MNNG-HOS and 143B cells. (**E**) Cells survival of OS cells before and after ESPN knockdown was detected by colony formation assay. (**F**) The overexpression efficiency of ESPN in OS cells (MG63) was detected by Western blot. (**G**, **H**) Scratch and (**I**) Transwell assays for cells migration and invasion ability. (**J**, **K**) The results of CCK-8 experiments and colony formation assay showed that ESPN gene overexpression increased the proliferation ability of MG63 cells. Data are expressed as the mean ± SD, scale bar = 50 μm, *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$

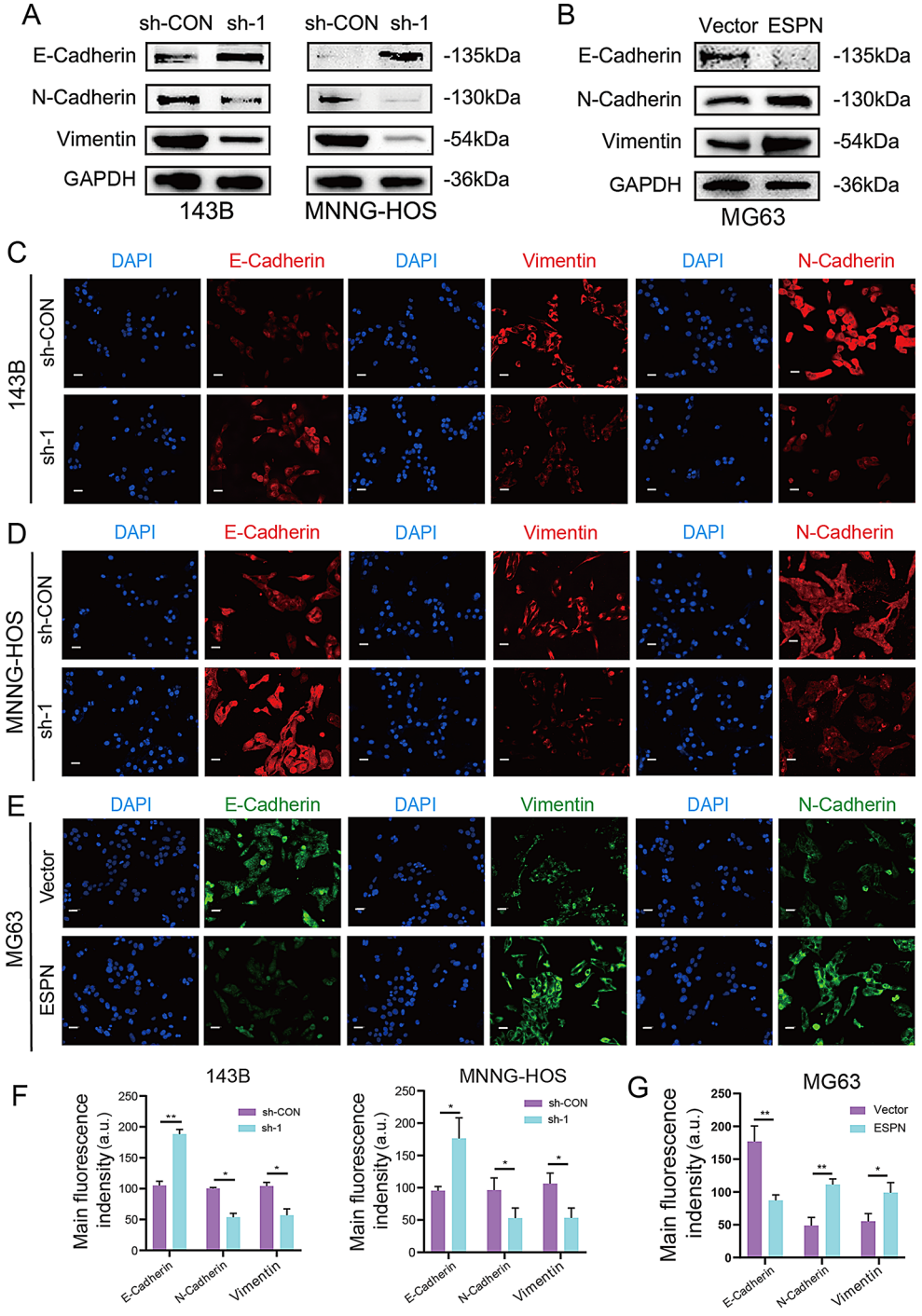

Fig. 3 ESPN accelerates EMT in OS cells (**A**, **B**) The effect of ESPN knockdown or overexpression on the expression of EMT-related marker protein in OS cell lines was examined using Western blot. (**C**, **D**) Immunofluorescence analysis of E-cadherin, N-cadherin, and Vimentin expression after treatment of 143B and MNNG-HOS cells with sh-CON or sh-1. DAPI is the blue color of nuclei. (**E**) Immunofluorescence analysis of E-cadherin, N-cadherin, and Vimentin expression after treatment of MG63 cells by Vector or ESPN. DAPI staining is shown in blue and indicates nuclei. (**F**, **G**). Immunofluorescence scoring of E-cadherin, N-cadherin and Vimentin. Data are expressed as the mean ± SD, scale bar = 20 μm, *$p < 0.05$, **$p < 0.01$

expression (Fig. 4E). In OS cells, ZEB1 knockdown partially reversed the EMT-promoting effect induced by forced ESPN expression (Fig. 4F). ZEB1 silencing also reversed the enhanced motility of MG63 cells with ESPN overexpression (Fig. 4G, H and Supplement Fig. 2G, H). Additionally, CCK-8 and colony formation assay demonstrated that ESPN influenced the proliferative capacity of OS cells through ZEB1 (Supplement Fig. 2I-K). Briefly,

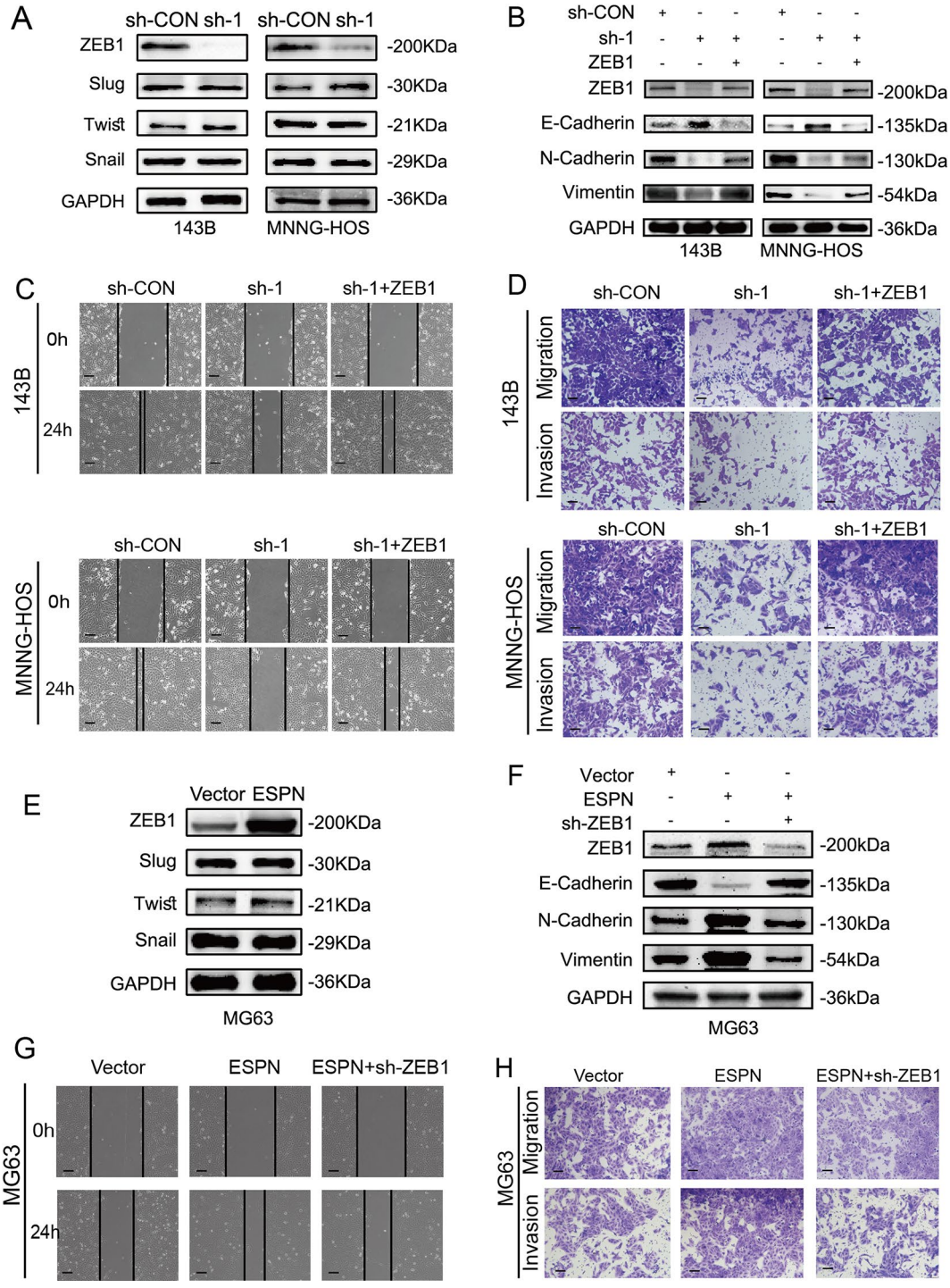

Fig. 4 ESPN enhances EMT by promoting ZEB1 expression (**A**) Effects of ESPN knockdown on ZEB1, Snail, Slug and Twist protein expression as assessed by Western blot. (**B**) The expression levels of ZEB1, E-cadherin, N-cadherin, and Vimentin proteins were analyzed in different groups (sh-CON, sh-1 and sh-1 + ZEB1) of 143B and MNNG-HOS cells. (**C**, **D**) Scratch, transwell and invasion assays were performed to analyze the effects of knockdown of ESPN as well as simultaneous overexpression of ZEB1 on OS migration and invasion. (**E**) Effects of ESPN overexpression on ZEB1, Snail, Slug and Twist expression as assessed by Western blot. (**F**) Expression of ZEB1, E-cadherin, N-cadherin and Vimentin protein was analyzed upon overexpression of ESPN and concurrent knockdown of ZEB1. (**G**, **H**) To analyze the effects of knockdown of ZEB1 and simultaneous overexpression of ESPN on OS migration and invasion, we performed scratch, transwell and invasion assays. Scale bar = 50 μm

ESPN affects the motility and proliferation of osteosarcoma cells by regulating ZEB1.

ESPN promotes OS cells migration and invasion through PI3K/AKT/mTOR/ZEB1 pathway

To elucidate the molecular mechanisms underlying ESPN-mediated EMT, we silenced the ESPN and evaluated the activation status of several key signaling pathways involved in regulating EMT. As shown in Supplement Fig. 3A-D and Fig. 5A, the PI3K/AKT/mTOR pathway stands out as the only one exhibiting significant alteration. The PI3K/AKT/mTOR signaling pathway regulates a wide range of cellular activities, including motility and proliferation, mTOR is frequently dysregulated in cancer, leading to EMT abnormalities [20–22]. Knockdown of ESPN specifically suppressed the phosphorylation of PI3K without affecting its total protein levels (Fig. 5A). Furthermore, molecular docking simulations using the HDOCK server (http://hdock.phys.hust.edu.cn/) predicted potential binding sites between ESPN and PI3K (Fig. 5B). To validate the interaction between ESPN and PI3K, co-immunoprecipitation (Co-IP) assays were performed in osteosarcoma cells using HA-tagged ESPN and MYC-tagged PI3K. The results show that PI3K effectively co-precipitates with ESPN, which provides strong evidence for a direct physical interaction between them (Fig. 5C). Meanwhile, ESPN suppression resulted in a significant reduction in phosphorylated AKT (p-AKT) and phosphorylated mTOR (p-mTOR) levels, while the total protein levels of AKT (t-AKT) and mTOR (t-mTOR) remained unchanged (Fig. 5A). Conversely, ESPN overexpression led to a marked increase in p-PI3K, p-AKT, and p-mTOR levels (Fig. 5F). These findings suggest that ESPN regulates the phosphorylation of PI3K by direct interaction with it and active the AKT/mTOR pathway. To further confirm whether ESPN regulates ZEB1 expression via the PI3K/AKT/mTOR pathway, we treated ESPN-deficient 143B cells with the mTOR agonist 3BDO. 3BDO restored the expression of p-mTOR and ZEB1 (Fig. 5D) and partially enhanced the motility of MNNG-HOS and 143B cells (Fig. 5E and Supplement Fig. 3E-H). Conversely, the addition of the mTOR inhibitor Rapamycin partially decreased the expression of both p-mTOR and ZEB1 in ESPN stably expressed MG63 cells (Fig. 5G). The motility of MG63 cells increased following ESPN overexpression but decreased with the addition of Rapamycin (Fig. 5H, I and Supplement Fig. 3I-K). In summary, these findings indicate that ESPN directly interacts with PI3K to activate the AKT/mTOR axis, thereby regulating ZEB1 expression, promoting osteosarcoma cell motility, and driving EMT progression.

ESPN promotes the translation of ZEB1 via mTOR/S6K/S6 pathway

The above studies indicate that the ESPN-mediated PI3K/AKT/mTOR signaling pathway can promote the translation of ZEB1, however, the underlying mechanism remains unknown. Numerous studies have shown that the mTOR/S6K signaling pathway is involved in a wide range of diseases and important biological processes including protein translation [23, 24]. Western blot analysis revealed that ESPN knockdown mediated a reduction in the levels of p-mTOR, p-S6K, and p-S6 proteins. Whereas overexpression of ESPN produces the opposite result (Fig. 6A, B). Next, we used the S6K inhibitor PF-4708671 to determine whether ESPN functions through the mTOR/S6K/S6 pathway. Endogenous overexpression of ESPN increased the expression of p-S6K, p-S6, and ZEB1, which were decreased by the introduction of PF-4708671 (Fig. 6B). Cells treated with PF-4708671 exhibited a partial reversal in motility as opposed to untreated cells (Fig. 6C-F). Consistently, the enhanced proliferation ability of OS cells induced by ESPN overexpression was reduced upon the addition of PF-4708671 (Fig. 6G-I). These data suggest that ESPN can promote ZEB1 protein expression via the PI3K/AKT/mTOR/S6K/S6 pathway, thereby promoting the motility and proliferation of OS cells in vitro.

ESPN promotes OS lung metastasis in vivo

To verify whether ESPN can promote OS metastasis in vivo by modulating ZEB1, we divided the 143B cells into three groups: control (sh-CON), low ESPN expression (sh-1), and high ZEB1 expression with low ESPN expression (sh-1 + ZEB1). These groups were injected into mice to establish a tail vein-lung metastasis model in BALB/C nude mice. After four weeks, the mice were sacrificed, and lung metastasis of OS cells was analyzed. The number of lung metastatic nodules in the sh-CON group was higher than in the sh-1 group, while the sh-1 + ZEB1 group showed more lung metastases than the sh-1 group (Fig. 7A, B). Furthermore, we divided MG63 cells into three groups: control (Vector), ESPN overexpression (ESPN), and high ESPN expression with ZEB1 knockdown (ESPN + sh-ZEB1). The results showed that the number of lung metastatic nodules in the ESPN group was higher than in the sh-CON group, while the ESPN + sh-ZEB1 group showed lower lung metastases than the ESPN group (Fig. 7C, D). Notably, we compared the expression of ESPN, p-S6K and ZEB1 in human OS situ tissue samples (Fig. 7E and Supplement Fig. 4A). We found a positive correlation between the expression pattern of ESPN, p-S6K and ZEB1 by IHC. These results strongly suggest that ESPN regulates ZEB1 and consequently activates OS migration through p-S6K in vivo.

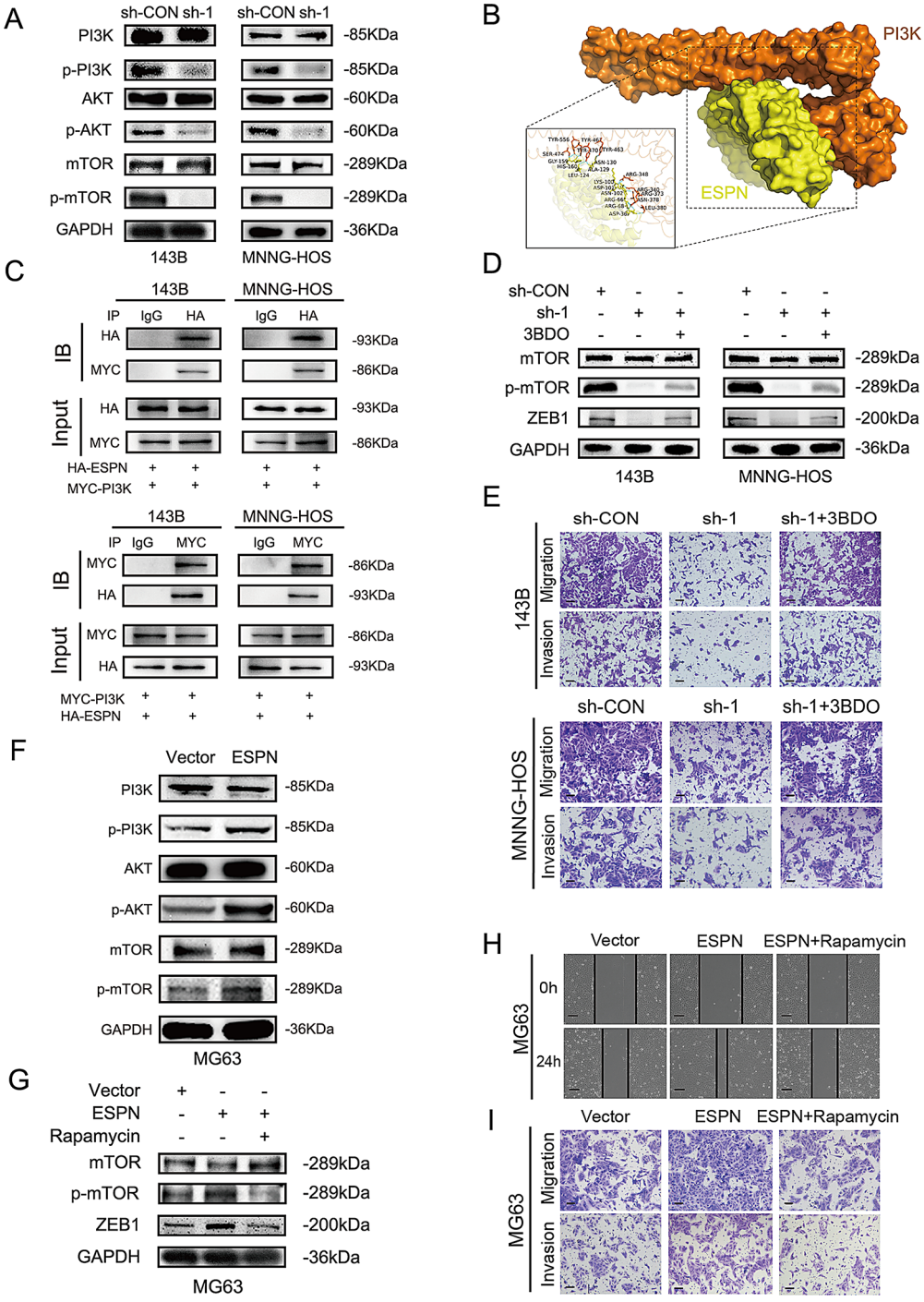

Fig. 5 ESPN promotes OS cells migration and invasion through PI3K/AKT/mTOR/ZEB1 pathway (**A**) Western blot analysis of phosphorylated and total PI3K, AKT, and mTOR protein expression in control and ESPN knockout OS cells. (**B**) The schematic diagram illustrates the binding interaction between ESPN and PI3K, with PI3K depicted in brown and ESPN represented in yellow. (**C**) Immunoprecipitation and Western blot analysis demonstrated the interaction between MYC-tagged PI3K and HA-tagged ESPN in 143B and MNNG-HOS cells. (**D**) The protein expression levels of mTOR, p-mTOR, and ZEB1 were detected in different groups (sh-CON, sh-1 and sh-1 + 3BDO) of 143B and MNNG-HOS cells. (**E**) The migration and invasion abilities of 143B and MNNG-HOS cells in different groups (sh-CON, sh-1 and sh-1 + 3BDO) were assessed using transwell and invasion assays. (**F**) Determination of PI3K, AKT and mTOR phosphorylated proteins and total protein expression before and after ESPN overexpression in OS cells by Western blot. (**G**) The protein expression levels of mTOR, p-mTOR, and ZEB1 were measured in different groups (Vector, ESPN and ESPN + Rapamycin) of MG63 cells. (**H**, **I**) The migration and invasion abilities of MG63 in different groups (Vector, ESPN and ESPN + Rapamycin) were assessed using transwell and invasion assays. These data represent three independent experiments. Scale bar = 50 μm

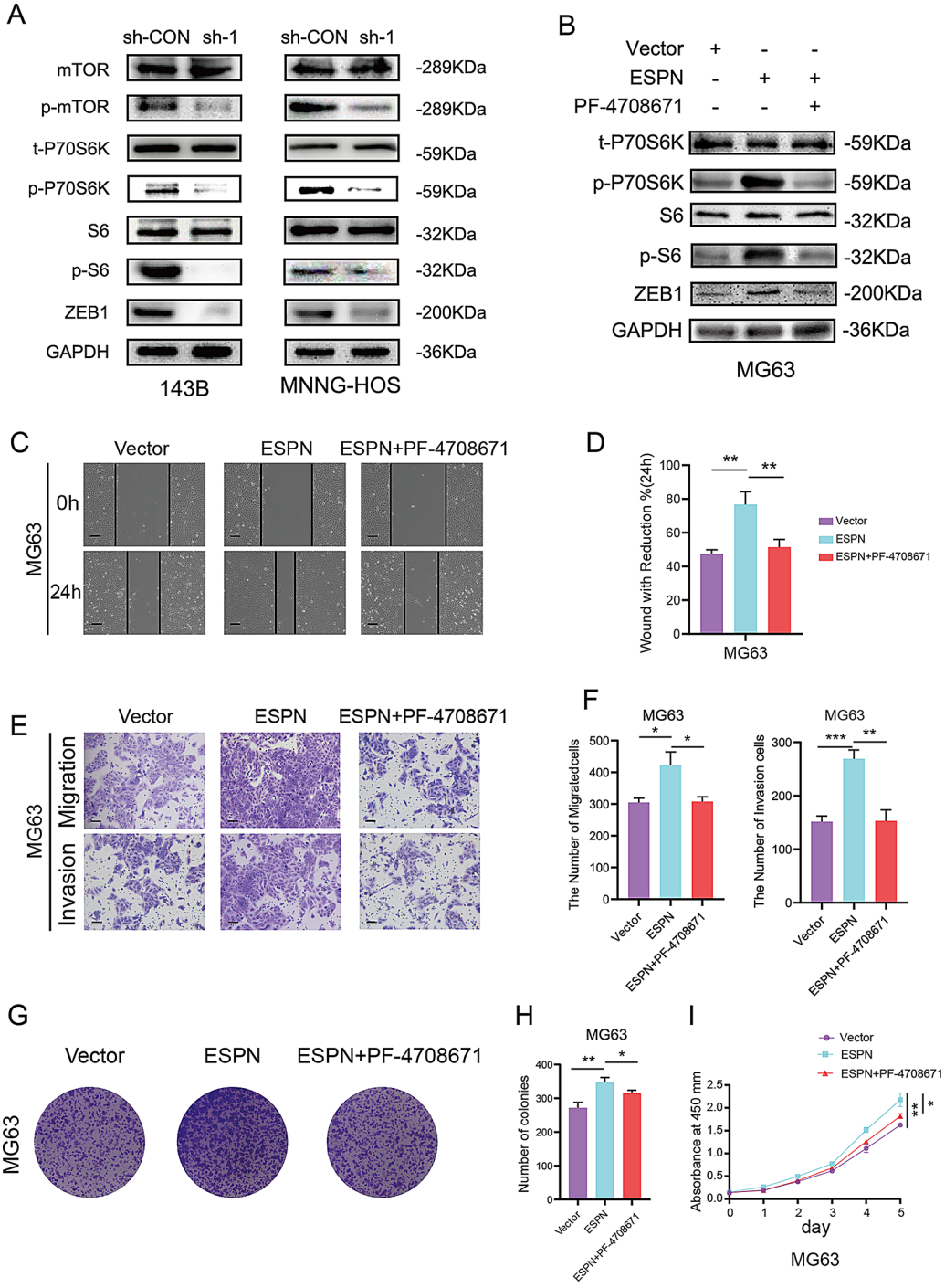

Fig. 6 ESPN promotes the translation of ZEB1 via mTOR/S6K/S6 pathway (**A**) 143B and MNNG-HOS cells were knockdown ESPN (sh-1), and the protein expression of t-mTOR, p-mTOR, t-p70S6K, p-p70S6K, S6, p-S6 and ZEB1 in the cells was detected by Western blot. (**B**) Overexpression of ESPN and co-culture of PF-4708671 in MG63 cells were detected by Western blot for protein expression of t-p70S6K, p-p70S6K, S6, p-S6 and ZEB1 in the cells. (**C**–**F**) MG63 cells underwent ESPN overexpression and were treated with the inhibitors, after which their metastasis and invasiveness were measured using the scratch, invasion and transwell assays. (**G**–**I**) MG63 cells underwent ESPN knockdown and were treated with the inhibitors, after which their viability was measured using the CCK-8 assay and colony formation assay. Data are expressed as the mean ± SD, Scale bar = 50 μm, *$p < 0.05$, **$p < 0.01$

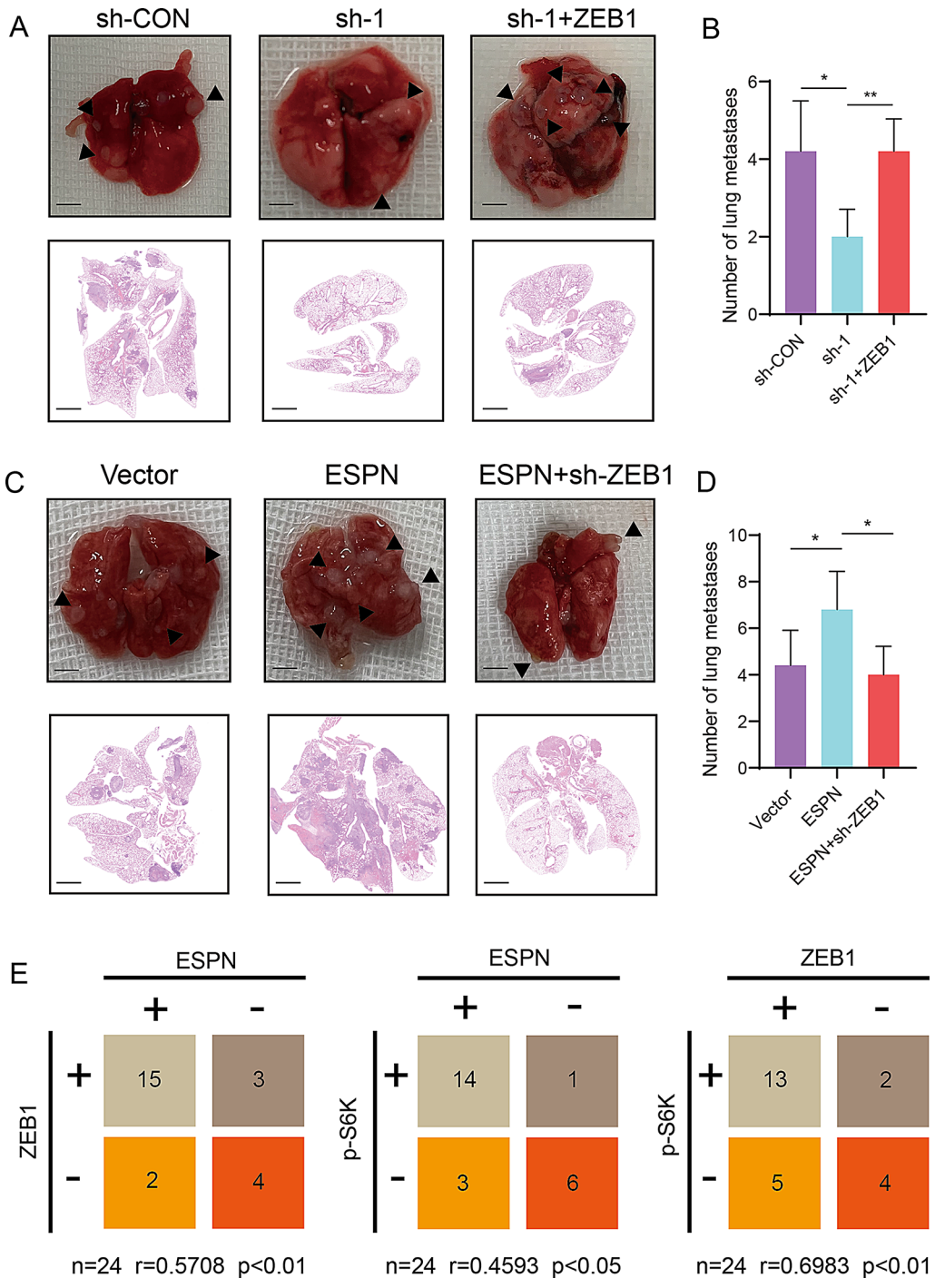

Fig. 7 ESPN promotes OS metastasis in vivo (**A**) A lung metastasis model was established in nude mice by injecting 143B cells from the control group (sh-CON), the ESPN knockdown group (sh-1), and the ESPN knockdown with ZEB1 overexpression group (sh-1 + ZEB1). Tail vein ($n = 5$/group). Top: Representative lung images. Bottom: Representative HE staining results. Pulmonary nodules are indicated by black triangles. Scale bar = 5 mm. (**B**) The number of metastatic pulmonary nodules in the specified group. (**C**) MG63 cells from different treatment groups (Vector, ESPN and ESPN + sh-ZEB1) were injected into the tail vein of nude mice to establish a lung metastasis model ($n = 5$/group). Top: Representative lung images; Bottom: Representative HE staining results. Pulmonary nodules are indicated by black triangles. Scale bar = 5 mm. (**D**) The number of metastatic pulmonary nodules in the specified group (Vector, ESPN and ESPN + sh-ZEB1). (**E**) Immunohistochemical analysis of ESPN, p-S6K and ZEB1 expression in osteosarcoma tissues. Data are expressed as the mean ± SD, $^{*}p < 0.05$, $^{**}p < 0.01$

Discussion

ESPN is associated with the development of various malignancies, and its abnormal alterations in actin have been implicated in neurodegenerative diseases [25]. In this study, we aimed to investigate the role of ESPN in the progression of OS. We observed a significant upregulation of ESPN in OS. Knockdown of ESPN suppressed the viability and motility of OS cells in vitro. Overexpression of ESPN had the opposite effect in vitro. Furthermore, stable high expression of ESPN promoted the formation of metastatic lung nodules, while stable reduction of endogenous ESPN levels decreased metastatic lung nodules. These findings indicate that ESPN plays a crucial role in OS progression.

The regulatory mechanisms by which ESPN promotes osteosarcoma metastasis have not been extensively studied. EMT involves a transition from epithelial to mesenchymal cell states, which is characterized by changes in the expression of epithelial and mesenchymal markers [26]. In OS cells, changes in the expression of mesenchymal and epithelial markers are associated with ESPN levels. These changes are often reflected through the regulation of EMT transcription factors (ZEB1, Slug, Snail, and Twist1) [27]. ZEB1 is commonly activated in various cancers and is involved in numerous cellular processes. For instance, ZEB1 is a determinant of anti-estrogen resistance in breast cancer [28], and promotes tumor metastasis in pancreatic cancer through YAP1 [29]. In addition, ZEB1 is upregulated in OS and regulates the occurrence of EMT in OS [30]. We observed that the reduction of ESPN weakened the translation of ZEB1 mRNA, which may explain the decreased ZEB1 protein expression. Alterations in ZEB1 expression further impact the levels of mesenchymal and epithelial markers and affect the EMT process.

EMT is a complex regulatory process involving multiple pathways. Emerging evidence has demonstrated that a complex network of signaling pathways, including TGF-β, Wnt, Notch, NF-κB, and PI3K/AKT/mTOR, plays a pivotal role in orchestrating the EMT during cancer progression [19, 31]. Following ESPN knockdown, we conducted a detailed analysis of several signaling pathways. Notably, only the PI3K/AKT/mTOR pathway exhibited significant changes, highlighting its critical involvement in the ESPN-mediated signaling axis. The PI3K/AKT/mTOR signaling pathway plays diverse roles in regulating various biological behaviors of tumor cells and is crucial in cancer development [32–34]. Zhao et al. found that histamine receptor H3 can block EMT process by inhibiting the conduction of PI3K/AKT/mTOR signaling pathway, thereby suppressing metastasis in human non-small cell lung cancer [35]. In Jin's study, miR-1224-5p promoted OS cell invasion through the PI3K/AKT/mTOR pathway [36]. Moreover, dual inhibitors of AKT and mTOR have shown encouraging results in clinical trials [37, 38]. We confirmed through database predictions and Co-IP assays that ESPN directly interacts with PI3K to regulate its phosphorylation, thereby activating the AKT/mTOR pathway. Based on these findings, we propose that ESPN modulates ZEB1 expression via the PI3K/AKT/mTOR pathway, thereby influencing EMT progression and metastasis in OS. Furthermore, the elevated expression of ESPN and ZEB1 holds prognostic significance, as it is associated with poor clinical outcomes.

To further elucidate how ESPN promotes ZEB1 expression via mTOR, we reviewed relevant literature and found that S6K is one of the substrates of mTOR, involved in regulating many critical cellular functions [39]. Phosphorylation of S6 occurs through the regulation of the mTOR/S6K signaling pathway [40]. Activated S6K further phosphorylates the S6 protein of the 40 S ribosomal subunit, promoting the translation of 5'TOP mRNA transcripts, which are primarily responsible for encoding key components of the protein translation machinery, thereby accelerating protein synthesis [41]. Activation of S6 has been shown to be involved in a range of physiological and pathological activities [42]. Our data indicate that ESPN induces the expression of ZEB1 by upregulating p-mTOR, p-S6K, and p-S6. Furthermore, it explains that the increase in ZEB1 expression is due to the activation of S6, which promotes ZEB1 translation, without affecting ZEB1 mRNA expression. IHC also confirmed that ESPN, p-S6K, and ZEB1 are positively correlated in OS tissues. These results suggest that ESPN regulates PI3K phosphorylation through direct interaction with PI3K, thereby activating the AKT/mTOR/S6K/S6 signaling pathway. Consequently, this promotes ZEB1 protein expression, contributing to the progression of OS and EMT.

Conclusions

In this study, ESPN was found to be increased in OS and is commonly correlated with poor prognosis. Its deficiency inhibited cell viability and motility, while its overexpression produced the opposite outcome. ESPN directly interacts with PI3K to active the AKT/mTOR/S6K/S6 signaling pathway, which in turn modulates ZEB1 mRNA translation, thereby influencing EMT. We also demonstrated in vivo that ESPN can regulate OS lung metastasis through ZEB1. Therefore, our findings suggest that ESPN may be a promising therapeutic target for OS, representing a potential new direction for OS treatment.

Abbreviations

OS	Osteosarcoma
OC	Osteochondroma
hFOB1.19	Human osteoblast cell line
IHC	Immunohistochemistry
CCK-8	Cell Counting Kit-8
qPCR	Quantitative real-time polymerase chain reaction

EMT	Epithelial-mesenchymal transition
p70S6K	p70 ribosomal protein S6 kinase
S6K	p70S6K
Co-IP	Co-immunoprecipitation

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12967-025-06500-8.

Supplementary Material 1: Fig. 1 (A) Knockdown efficiency of ESPN gene in OS cells detected by qRT-PCR. (B) Statistical analysis of the effect of ESPN knockdown on the migration ability of OS cells based on the scratch assay. (C, D) Statistical analysis of the effect of ESPN knockdown on the migration and invasion ability of OS cells based on the transwell and invasion assay. (E) The effect of ESPN knockdown on the proliferative ability of OS cells using clone formation experiments. (F) The effect of ESPN overexpression on the proliferative capacity of OS cells was analyzed using clone formation experiments. (G) The effect of ESPN overexpression on the metastatic and invasive ability of OS cells was analyzed based on data from transwell and invasion assays. Data are expressed as mean ± standard deviation. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Supplementary Material 2: Fig. 2 (A) PCR analysis of the effect of knockdown of ESPN on EMT transcription factors in osteosarcoma cells. (B) Histogram representation of quantitative Western blot analysis showing expression changes of EMT-related transcription factors after ESPN knockdown. (C) R2 database analysis of the effect of ZEB1 on the prognosis of overall survival of patients with osteosarcoma. (D-F) Scratch, transwell and invasion assays were performed to analyze the migration and invasion abilities of 143B and MNNG-HOS cells with different treatments (sh-CON, sh-1 and sh-1 + ZEB1). (G, H) Scratch, transwell and invasion assays were performed to analyze the migration and invasion abilities of MG63 with different treatments (Vector, ESPN and ESPN + sh-ZEB1). (I, J) CCK-8 and clone formation experiments were performed to analyze the proliferation of 143B and MNNG-HOS cells using different treatments (sh-CON, sh-1 and sh-1 + ZEB1). (K) Analysis of the results of clone formation experiments using cells with different treatments. Data are expressed as mean ± standard deviation. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Supplementary Material 3: Fig. 3 (A-D) Western-blot detection of the effect of knockdown of ESPN on Notch, TGF-β, Wnt/β-Catenin and NF-κB signaling pathways. (E) The migration abilities of 143B and MNNG-HOS cells in different groups (sh-CON, sh-1 and sh-1 + 3BDO) were assessed using scratch experiments. (F) Assessment of the migration abilities of OS cells (143B and MNNG-HOS) in different groups (sh-CON, sh-1 and sh-1 + 3BDO) with scratch experiments. (G, H) Assessment of the migration and invasion abilities of OS cells (143B and MNNG-HOS) in different groups (sh-CON, sh-1 and sh-1 + 3BDO) with transwell and invasion experiments. (I) Assessment of the migration abilities of MG63 in different groups (Vector, ESPN and ESPN + Rapamycin) by scratch experiment. (J, K) Assessment of the migration and invasion abilities of MG63 in different groups (Vector, ESPN and ESPN + Rapamycin) by transwell and invasion experiments. Data are expressed as mean ± standard deviation. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$

Supplementary Material 4: Fig. 4 (A) Landmark images of immunohistochemistry of consecutive in situ osteosarcoma tissues. Scale bar = 500 μm

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Author contributions
RK, HY and HY conducted experiments, analyzed the data and wrote the paper. YF, YQ and DZ participated in the design of the study. Others participated in the experiments and aided. All authors read and approved of the final manuscript.

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Data availability
The datasets generated and/or analyzed during this study are available upon request from the corresponding author.

Declarations

Ethical approval
This study was approved by the research medical ethics committee of the Affiliated Hospital of the Nanjing Medical University Animal Protection and Use Committee. Informed Consent: N/A. Registry and the Registration No. of the study/trial: N/A. Animal Studies: N/A.

Consent for publication
All authors agreed on the manuscript.

Competing interests
The authors have no competing interests to declare.

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