Transcription factor HOXB2 upregulates NUSAP1 to promote the proliferation, invasion and migration of nephroblastoma cells via the PI3K/Akt signaling pathway

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Abstract. The transcription factor homeobox protein Hox-B2 (HOXB2) and its downstream factor nucleolar and spindle-associated protein 1 (NUSAP1) play important regulatory roles in cell proliferation, invasion and migration. However, their effects and specific mechanisms in nephroblastoma have not been previously investigated, to the best of our knowledge. Therefore, in the present study, the mRNA and protein expression levels of HOXB2 and NUSAP1 were determined in nephroblastoma cells using reverse transcription-quantitative PCR and western blot analyses, respectively. Furthermore, cell transfection experiments were carried out to knock down NUSAP1 and overexpress HOXB2 in nephroblastoma cell lines. The proliferative, invasive and migratory abilities of nephroblastoma cells were assessed by MTT, EdU, colony formation, wound healing and Transwell assays. In addition, the JASPAR website was used to predict the association between HOXB2 and NUSAP1, which was further verified by dual-luciferase reporter and chromatin immunoprecipitation assays. Finally, the expression levels of the PI3K/Akt signaling pathway-related proteins were measured by western blot analysis. The results showed that the expression of NUSAP1 was abnormally upregulated in nephroblastoma cell lines. However, NUSAP1 silencing attenuated the proliferation, invasion and migration abilities of nephroblastoma cells. The results also suggested that HOXB2 could transcriptionally activate NUSAP1. Therefore, HOXB2 overexpression abrogated the inhibitory effect of NUSAP1 silencing on the proliferation and metastasis of nephroblastoma cells, possibly via the PI3K/Akt signaling pathway. The aforementioned findings indicated that HOXB2 may upregulate NUSAP1 to promote the proliferation, invasion and migration of nephroblastoma cells via the PI3K/Akt signaling pathway.

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Introduction

Nephroblastoma is the most common malignancy of the urinary tract in children, accounting for ~8% of all pediatric solid tumors (1). Nephroblastoma most commonly occurs at the ages of 1-3 years and affects ~1 in 10,000 individuals (2). Nephroblastoma, which most commonly manifests as an abdominal mass, is associated with high morbidity and mortality rates and adversely affects the quality of life of the patients (3). Combination treatment with surgery, radiotherapy and chemotherapy has greatly improved the survival rate of patients with nephroblastoma (4). Currently, the 5-year overall survival rate of patients with nephroblastoma is ~85%. However, due to the insidious onset and rapid progression of the disease, 15% of all children have a poor prognosis (5). Therefore, identifying novel diagnostic markers and treatment approaches for children with nephroblastoma is crucial.

Transcription factors (TF) are proteins that can effectively bind upstream of particular genes, thus regulating their expression and driving a series of cellular processes (6). It has been reported that TFs serve a central role in the development of life, while their inactivation, which determines cell fate, is considered as an important step in tumorigenesis (7). Homeobox (HOX) genes are involved in regulating cell differentiation and morphological development and are therefore at the highest level of the genetic hierarchy (8). Homeobox protein Hox-B2 (HOXB2) is a member of the HOX family. A previous study demonstrated that HOXB2 served an important role in the proliferation, invasion and migration of ovarian cancer cells (9). In addition, microRNA-139-5p attenuated the proliferation of lung cancer cells via targeting HOXB2 (10). Furthermore, another study revealed that HOXB2 could promote the apoptosis of gastric cancer cells and enhance their sensitivity to cisplatin (10). Jing *et al* (11) demonstrated that HOXB2 and forkhead box C1 (FOXC1) were upregulated in nephroblastoma tissues, while their expression was closely

associated with tumor stage and lymph node metastasis. These findings suggested that HOXB2 and FOXC1 could synergistically promote the development of malignant tumors via enhancing the proliferative and migratory potential of nephroblastoma cells (11). However, the specific mechanism underlying the effects of HOXB2 on the proliferation and migration of nephroblastoma cells remains unclear.

In the present study, the binding potential of HOXB2 on the nucleolar spindle-associated protein 1 (NUSAP1) promoter was predicted using the JASPAR database. NUSAP1, a microtubule-binding protein, is highly conserved in humans and serves an indispensable role in mitosis via regulating the proper assembly of the spindle and the formation of chromosomes (12,13). It has been reported that, in normal tissues, the expression of NUSAP1 is tightly regulated; however, when NUSAP1 is abnormally upregulated, cell mitosis cannot be terminated, resulting in infinite cell proliferation (14). This finding could explain why the expression of NUSAP1 is abnormally elevated in cancer. Another study analyzed the gene expression profile in nephroblastoma using the Target Data Matrix database and showed that NUSAP1 was significantly upregulated (15). However, to the best of our knowledge, the role of NUSAP1 in nephroblastoma has not been previously reported.

Therefore, the current study aimed to investigate regulatory mechanisms in terms of TF and to predict significant regulatory genes involved in specific signaling pathways, in order to uncover the mechanism underlying the effect of HOXB2 and its downstream factor NUSAP1 in nephroblastoma, thus providing novel targets for the diagnosis and treatment of nephroblastoma.

Materials and methods

Cell culture. The nephroblastoma cell lines, GHINK-1, WiT49, 17-94 and HFWT, and 293T cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C in a 5% CO_2 incubator.

Database. The association between NUSAP1 and HOXB2 was predicted by bioinformatics analysis using the JASPAR database (jaspar.genereg.net).

Reverse transcription-quantitative PCR (RT-qPCR) analysis. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was then reverse transcribed into cDNA using MMLV reverse transcriptase (Promega Corporation) according to the manufacturer's specifications. qPCR analysis was carried out using SYBR Green mix (Thermo Fisher Scientific, Inc.). The PCR thermocycling conditions applied were as follows: Initial holding period at 95˚C for 30 sec, followed by 40 cycles of 94˚C for 5 sec and 60˚C for 30 sec. GAPDH served as an internal reference gene. The primer sequences used were as follows: NUSAP1 forward, 5'-CGTCCCCTCAACTATGAACCAC-3' and reverse, 5'-GCGTTTCTTCCGTTGCTCTT-3'; HOXB2 forward, 5'-CGCCAGGATTCACCTTTCCTT-3' and reverse, 5'-CCCTGTAGGCTAGGGGAGAG-3'; and GAPDH forward, 5'-AGAAGGCTGGGGCTCATTTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'. The quantification was performed using the $2^{-\Delta\Delta Cq}$ method (16).

Western blot analysis. Cells were lysed with RIPA buffer (Beyotime Institute of Biotechnology) with phosphatase inhibitor AEBSF (Beyotime Institute of Biotechnology) on ice for 30 mins. Following centrifugation at 350 x g for 20 min at 4˚C, a BCA protein assay kit (Sigma-Aldrich; Merck KGaA) was utilized to quantify the protein concentration. Proteins (40 μg/lane) were separated by 10% SDS-PAGE and were then transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). Following blocking with 5% skimmed milk for 1.5 h at room temperature, the membranes were incubated with primary antibodies against NUSAP1 (1:1,000; cat. no. ab137230), HOXB2 (1:1,000; cat. no. ab220390), phosphorylated (p)-PI3K (1:1,000; cat. no. ab191606), p-Akt (1:1,000; cat. no. ab38449), E-cadherin (1:1,000; cat. no. ab40772), N-cadherin (1:1,000; cat. no. ab76011), Vimentin (1:1,000; cat. no. ab92547), PI3K (1:1,000; cat. no. ab32089), Akt (1:1,000; cat. no. ab18785) and GAPDH (1:1,000; cat. no. ab8245; all from Abcam) at 4˚C overnight. The next day, the membranes were washed and incubated with the corresponding secondary antibodies (1:5,000; cat. no. ab150077; Abcam) at room temperature for 1 h. For p-PI3K and p-Akt, after the detection of PI3K and Akt, the same membrane was washed with stripping buffer (cat. no. P0025N; Beyotime Institute of Biotechnology), and the aforementioned protein detection steps were repeated to detect the expression of p-PI3K and p-Akt. Protein signals were detected using ECL detection kit (Amersham; Cytiva) and semi-quantitative analysis was conducted using ImageJ software (version 1.8.0; National Institutes of Health).

Cell transfection. HFWT cells were cultured in a 6-well plate at a density of $1x10^6$ cells/well at 37˚C in a 5% CO_2 incubator for 24 h. The lentiviral vectors packaging short hairpin RNA (shRNA) NUSAP1 (sh-NUSAP1), negative control shRNA (sh-NC), HOXB2 overexpression plasmid (Ov-HOXB2) and Ov-NC were obtained from Shanghai GeneChem Co., Ltd., and were then transduced into cells at 20 nM using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The transfection efficiency was verified by RT-qPCR and western blot analyses after 48 h.

MTT assay. Following culture for 48 h at 37˚C in a 5% CO_2 incubator, HFWT cells in each well at a density of $8x10^3$ cells/well were supplemented with 20 μl MTT solution and incubated for an additional 3 h. Subsequently, the supernatant was removed and 200 μl DMSO was added into each well to dissolve formazan crystals. The absorbance at 490 nm was detected using a microplate reader (Multiskan Sky; Thermo Fisher Scientific, Inc.).

EdU staining. Cell proliferation was assessed using the Cell-Light EdU DNA Cell Proliferation kit (Guangzhou RiboBio Co., Ltd.) according to the manufacturer's protocol. Briefly, following transfection, the HFWT cells were stained with fluorescent-labeled EdU for 2 h at room temperature. Subsequently, cells were fixed in 4% ethanol for 30 min at room temperature and were then stained with Apollo

reaction buffer liquid at 37˚C for 30 min in the dark. Image acquisition was performed using a laser confocal microscope (magnification, x200).

Colony formation assay. Transfected HFWT cells were cultured normally in a 6-well plate at a density of $1x10^3$ cells/well. Following incubation for 2 weeks, cells were fixed with methanol for 15 min at room temperature and stained with 0.1% crystal violet for 20 mins at room temperature. Finally, the visible colonies with a diameter >0.5 mm were counted using ImageJ software (version 1.8.0; National Institutes of Health) and images were captured under a microscope.

Wound healing assay. For wound healing assays, HFWT cells were grown to 100% confluence in 6-well plates after transfection. Subsequently, two linear wounds were created in each well by scratching the monolayer with a sterile pipette tip. The wells were then washed with PBS and cells were cultured in serum-free DMEM for 24 h. To assess cell migration, images of the same areas in the wells were captured at 0 and 24 h using a light microscope following wounding. Wound healing was evaluated by measuring the total surface area covered by the cells.

Transwell assay. To assess cell invasion, 24-well Transwell plates (Corning, Inc.) with 8-μm pore inserts were coated with Matrigel (BD Biosciences) at 37˚C for 30 min. The transfected HFWT cells ($5x10^5$ cells/well) in serum-free DMEM were seeded onto the Matrigel-coated upper chamber, while the lower chamber was supplemented with DMEM containing 10% FBS. Following incubation for 24 h at 37˚C, the cells on the upper chamber were gently removed with cotton swabs. The invading cells in the lower chamber were fixed in alcohol for 15 min and stained with 0.1% crystal violet solution for 10 min at room temperature. Finally, the cells were observed under an optical microscope (Olympus Corporation; magnification, x100).

Dual-luciferase reporter assay. To generate the luciferase construct, 0.5 μg vectors containing the 3'-untranslated region (UTR) of wild-type (WT) NUSAP1 or mutant (MUT) 3'-UTR NUSAP1using a QuickMutation kit (Beyotime Institute of Biotechnology), with control vector or HOXB2 overexpression vector and pMIR-*Renilla* vector (Shanghai GeneChem Co., Ltd) were synthesized and cloned into GV272 vectors by GeneChem (China). HFWT cells were co-transfected using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) with WT or MUT 3'-UTR of NUSAP1 (NUSAP1-WT or NUSAP1-MUT, respectively) and Ov-HOXB2 or Ov-NC for 48 h. A site directed mutagenesis kit was used to mutant 3'-untranslated region of NUSAP1. Following transfection, the relative luciferase activity was determined after 48h using the Dual-Luciferase Reporter Assay kit (Promega Corporation), according to the manufacturer's instructions. *Renilla* luciferase activity was used to normalize the firefly luciferase activity.

Chromatin immunoprecipitation (ChIP) assay. The ChIP assay was carried out using the Chromatin Immunoprecipitation Assay Kit (MilliporeSigma), according to the manufacturer's instructions. Briefly, the cells were lysed with 100 μl SDS lysis buffer (Beyotime Institute of Biotechnology) and 500 μl of ChIP sonication nuclear lysis buffer was added to cell particles and centrifuged at 1,000 x g at 4˚C for 5 mins. Then, 10 μg fragmented chromatin was incubated with 2 μg anti-IgG (cat. no. A-11031) and 5 μg anti-HOXB2 (cat. no. PA5-101640) antibodies (1:500; both Thermo Fisher Scientific, Inc.). Subsequently, 20 μl protein A+G agarose beads (Beyotime Institute of Biotechnology) was added and incubated for another 4 h at 4˚C. After the supernatant was removed, the collected agarose beads were washed five times with 100 μl PBS (0.01 M; pH 7.4) and denatured by boiling for 5 min. Total RNA was then subjected to reverse transcription to cDNA using SMART MMLC Reverse Transcriptase (Takara Biotechnology Co., Ltd.). The recruited DNA was then subjected to PCR using primers detecting the distal enhancer regions of NUSAP1 using an ABI 7500 system (Thermo Fisher Scientific, Inc.). Subsequently, the PCR products were electrophoresed in 1% agarose gel, stained in ethidium bromide for ~15 min, rinsed with water and photographed by gel imaging system. Gel images and positive bands with average optical density values were analyzed by Gel optical density analysis software Gel pro4.0. The resulting data were normalized to housekeeping gene β-actin.

Statistical analysis. All data are expressed as the mean ± SD of three independent experimental repeats. All statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, Inc.). The differences between multiple groups were compared by one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

NUSAP1 knockdown attenuates the proliferation of nephroblastoma cells. The mRNA and protein expression levels of NUSAP1 in nephroblastoma cells were detected via RT-qPCR and western blot analyses, respectively. The results showed that the expression of NUSAP1 was significantly increased in nephroblastoma cell lines compared with 293T cells (Fig. 1A and B). The expression of NUSAP1 was more notably increased in HFWT cells. Therefore, HFWT cells were used for the subsequent experiments. Subsequently, the expression of NUSAP1 was knocked down in HFWT cells following cell transfection with sh-NUSAP1 and the transfection efficiency was evaluated using RT-qPCR and western blot analysis. As shown in Fig. 1C and D, the expression of NUSAP4 was significantly decreased in cells transfected with shRNA-NUSAP1#2 and this shRNA clone was therefore used for the subsequent experiments. To evaluate cell viability, cells were first divided into the control, sh-NC and sh-NUSAP1 groups followed by MTT assay. The results showed that cell viability was significantly reduced in the sh-NUSAP1 group compared with the sh-NC group (Fig. 1E). Cell proliferation was assessed using colony formation assay. As shown in Fig. 1F, compared with the sh-NC group, the proliferative ability of cells was significantly decreased in the sh-NAUSAP1 group. The results of EdU staining were consistent with those obtained with the colony formation assay (Fig. 1G). The

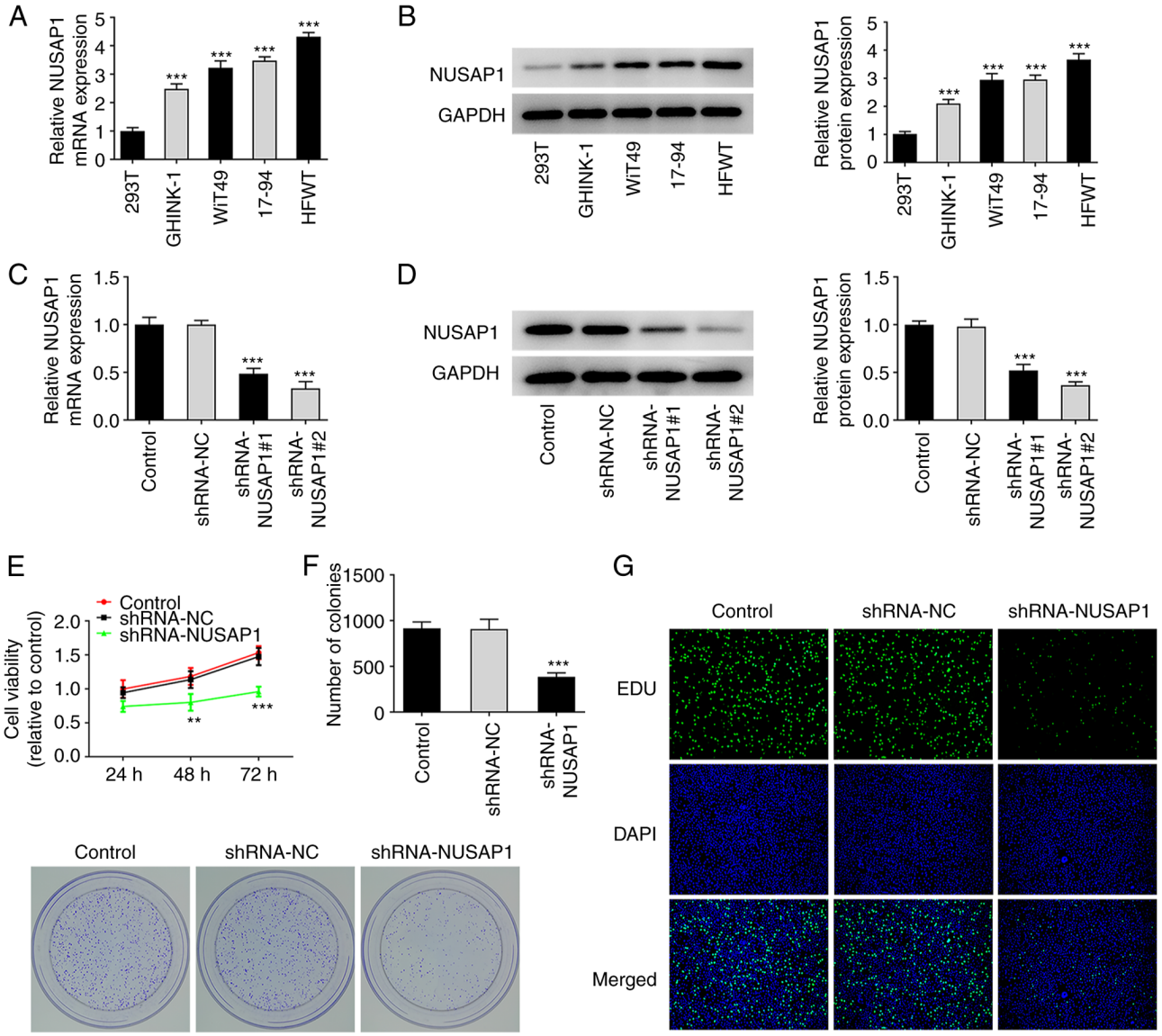

Figure 1. Knockdown of NUSAP1 inhibits the proliferation of nephroblastoma cells. (A) RT-qPCR and (B) western blotting were performed to detect the expression of NUSAP1 in nephroblastoma cell lines. ***P<0.001 vs. 293T. (C) RT-qPCR and (D) western blotting were performed to detect the expression of NUSAP1 after cell transfection. (E) MTT was conducted to detect cell viability. (F) Colony formation and (G) EdU staining assays were performed to detect cell proliferation. **P<0.01, ***P<0.001 vs. shRNA-NC. NUSAP1, nucleolar and spindle-associated protein 1; RT-qPCR, reverse transcription-quantitative PCR; shRNA, short hairpin RNA; NC, negative control.

aforementioned findings suggested that NUSAP1 silencing could attenuate the proliferation of nephroblastoma cells.

NUSAP1 silencing inhibits the metastatic potential of nephroblastoma cells. Subsequently, wound healing and Transwell assays were carried out to evaluate the migratory and invasive abilities of nephroblastoma cells, respectively. The results demonstrated that the migratory and invasive abilities of nephroblastoma cells were notably decreased in the sh-NAUSAP1 group compared with the sh-NC group (Fig. 2A and B). Subsequently, western blot analysis was performed to detect the expression levels of epithelial-to-mesenchymal transition (EMT)-related proteins. The analysis showed that, compared with cells transfected with sh-NC, the expression of E-cadherin was significantly increased, while that of N-cadherin and vimentin was notably reduced in cells in the sh-NUSAP1 group (Fig. 2C). Therefore, it was hypothesized that NUSAP1 silencing could inhibit the metastasis of nephroblastoma cells.

TF HOXB2 activates NUSAP1. Bioinformatics analysis using the JASPAR database predicted a HOXB2 binding site on the NUSAP1 gene promoter (Fig. 3A and B). Subsequently, the expression of HOXB2 in nephroblastoma cells was assessed using RT-qPCR and western blot analyses. It was observed that HOXB2 was significantly upregulated in HFWT cells compared with 293T cells (Fig. 3C and D). Then, transfection experiments were performed to overexpress HOXB2 and the transfection efficiency was also determined. HOXB2 was significantly upregulated in the Ov-HOXB2 group compared with the Ov-NC group (Fig. 3E and F). The interaction between HOXB2 and NUSAP1 was verified by dual-luciferase reporter assay (Fig. 3G), and was further confirmed using a ChIP assay (Fig. 3H). In addition, the expression

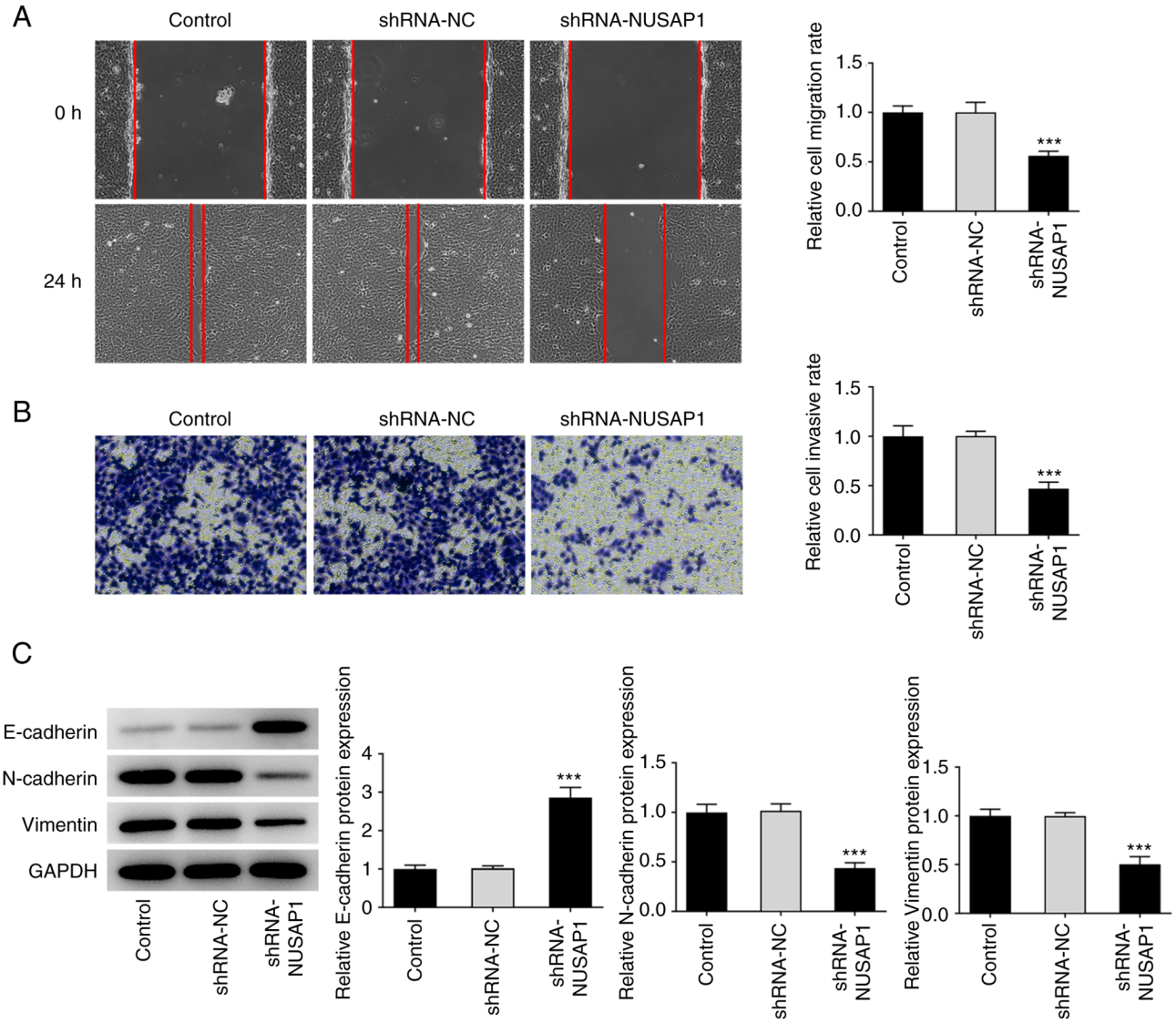

Figure 2. Knockdown of NUSAP1 inhibits metastatic potential of nephroblastoma cells. (A) Wound healing assay was performed to detect cell migration. (B) Transwell assay was performed to detect cell invasion. Magnification, x200. (C) Western blotting was conducted to detect the expression of metastasis-related proteins. ***P<0.001 vs. shRNA-NC. NUSAP1, nucleolar and spindle-associated protein 1; shRNA, short hairpin RNA; NC, negative control.

of NUSAP1 in the Ov-HOXB2 group was significantly increased after overexpression of HOXB2 compared with the Ov-NC group (Fig. 3I). Subsequently, cells were divided into the control, sh-NC, sh-NUSAP1, sh-NUSAP1 + Ov-NC and sh-NUSAP1 + Ov-HOXB2 groups and the expression of NUSAP1 was then determined. The results showed that HOXB2 overexpression could reverse the sh-NUSAP1-mediated reduced NUSAP1 expression (Fig. 3J and K). These findings indicated that HOXB2 could promote the expression of NUSAP1.

HOXB2 overexpression reverses the inhibitory effect of NUSAP1 silencing on the proliferation and metastatic potential of nephroblastoma cells. MTT, EdU and colony formation assays were performed to evaluate cell proliferation. Therefore, compared with the sh-NUSAP1 + Ov-NC group, the proliferative ability of nephroblastoma cells in the sh-NUSAP1 + Ov-HOXB2 group was significantly enhanced (Fig. 4A-D). Additionally, wound healing and Transwell assays showed that compared with the sh-NUSAP1 + Ov-NC group, the invasive and migratory abilities of nephroblastoma cells in the sh-NUSAP1 + Ov-HOXB2 group were also significantly increased (Fig. 5A and B). To assess the expression levels of EMT-related proteins, western blot analysis was carried out. The analysis showed that compared with the sh-NUSAP1 + Ov-NC group, the expression of E-cadherin was decreased, while that of N-cadherin and Vimentin was increased in the sh-NUSAP1 + Ov-HOXB2 group (Fig. 5C). These findings suggested that HOXB2 overexpression could reverse the inhibitory effect of NUSAP1 knockdown on the proliferation and metastasis of nephroblastoma cells.

HOXB2 overexpression reverses the inhibitory effect of NUSAP1 silencing on the proliferation and metastatic potential of nephroblastoma cells via the PI3K/Akt signaling pathway. Western blot analysis revealed that the expression levels of the PI3K/Akt pathway-related proteins were abnormally altered. Therefore, the expression levels

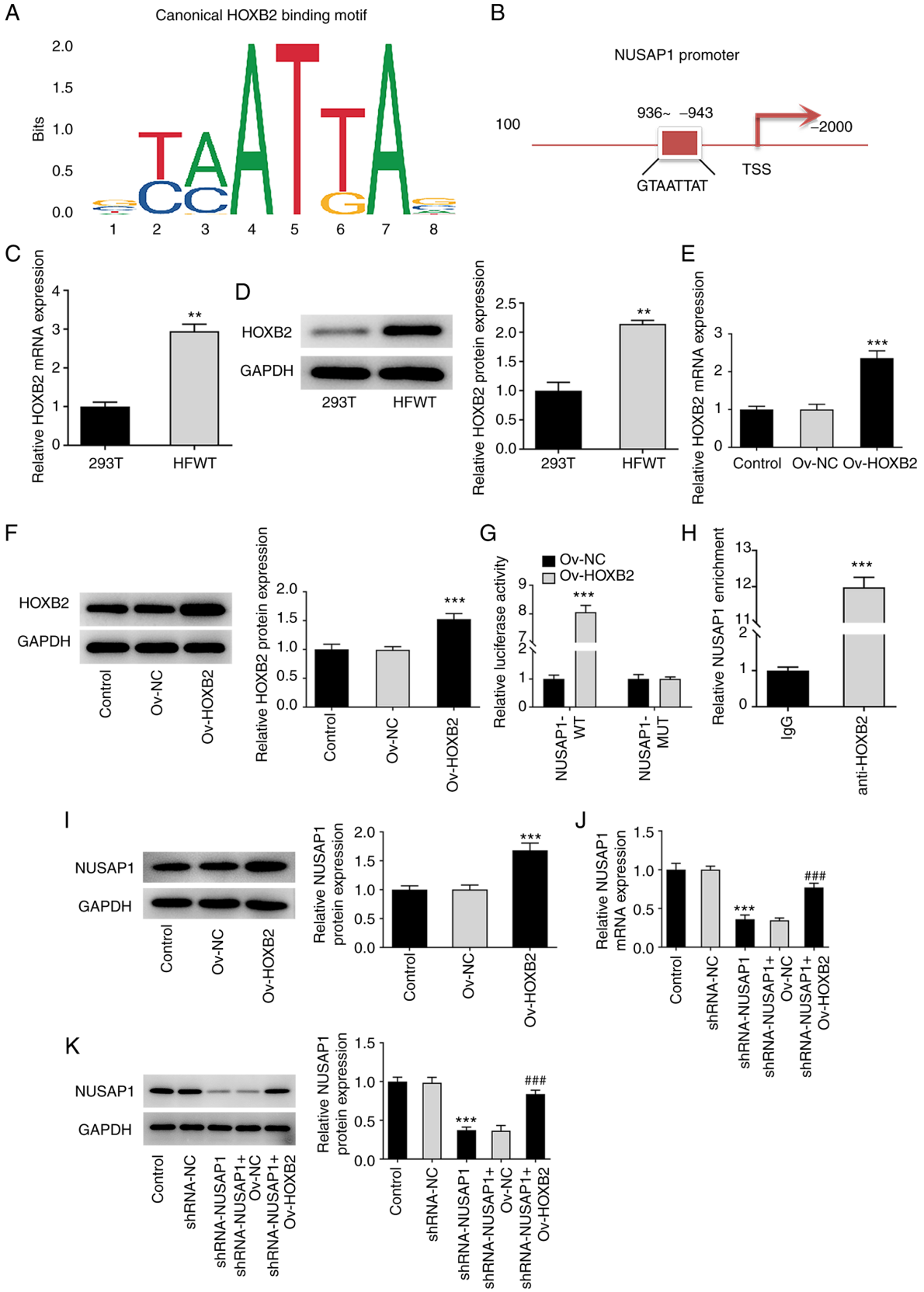

Figure 3. Transcription factor HOXB2 specifically activates NUSAP1. (A and B) JASPAR website was used to predict the binding of HOXB2 and NUSAP1. (C) RT-qPCR and (D) western blotting was performed to detect the expression of HOXB2 in nephroblastoma cell lines. **P<0.001, ***P<0.001 vs. 293T. (E) RT-qPCR and (F) western blotting was performed to detect the expression of HOXB2 in nephroblastoma cell lines after overexpression of HOXB2. (G) The dual-luciferase reporter assay confirmed the relationship between HOXB2 and NUSAP1. (H) Chromatin immunoprecipitation assay confirmed the relationship between HOXB2 and NUSAP1. (I) Western blotting was conducted to detect the expression of NUSAP1 in nephroblastoma cells after overexpression of HOXB2. ***P<0.001 vs. Ov-NC. (J) RT-qPCR and (K) western blotting were performed to detect the expression of NUSAP1 in nephroblastoma cells after cell transfections. ***P<0.001 vs. shRNA-NC; $^{###}$P<0.001 vs. shRNA-NUSAP1 + Ov-NC. HOXB2, homeobox protein Hox-B2; NUSAP1, nucleolar and spindle-associated protein 1; RT-qPCR, reverse transcription-quantitative PCR; Ov, overexpression vector; shRNA, short hairpin RNA; NC, negative control; WT, wild-type; MUT, mutant.

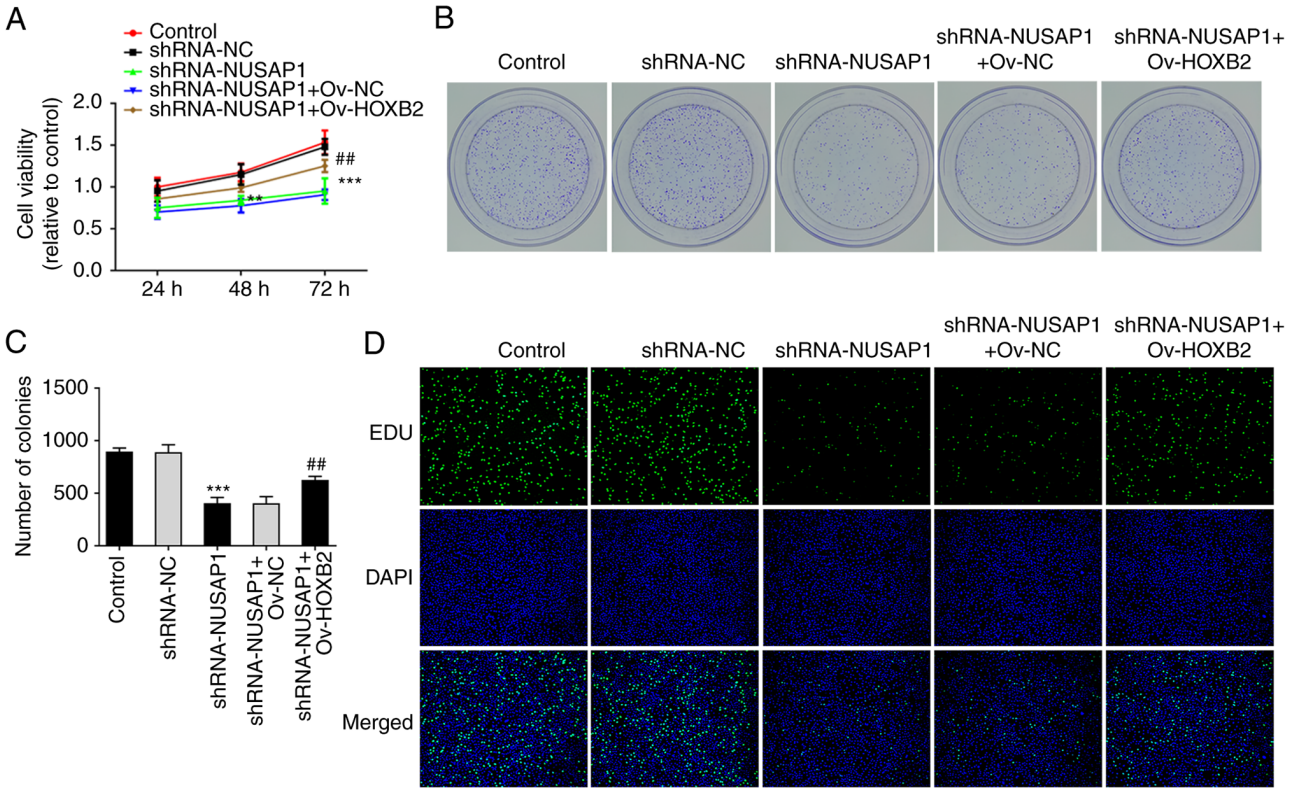

Figure 4. Overexpression of HOXB2 reverses the inhibitory effect of NUSAP1 knockdown on the proliferation of nephroblastoma cells. (A) MTT was performed to detect cell viability. (B) Colony formation assay was conducted to detect cell proliferation. (C) Statistical analysis of the results presented in (B). (D) EdU staining was performed to detect cell proliferation. **P<0.01, ***P<0.001 vs. shRNA-NC; $^{\#\#}$P<0.01, vs. shRNA-NUSAP1 + Ov-NC. HOXB2, homeobox protein Hox-B2; NUSAP1, nucleolar and spindle-associated protein 1; Ov, overexpression vector; shRNA, short hairpin RNA; NC, negative control.

of p-PI3K and p-Akt were significantly decreased in the sh-NUSAP1 group compared with the shRNA-NC group. By contrast, p-PI3K and p-Akt were significantly upregulated in the sh-NUSAP1 + Ov-HOXB2 group compared with the sh-NUSAP1 + Ov-NC group (Fig. 6). The aforementioned findings suggested that HOXB2 overexpression could reverse the inhibitory effect of NUSAP1 silencing on the proliferation and metastasis of nephroblastoma cells, possibly via the PI3K/Akt signaling pathway.

Discussion

Nephroblastoma is a common malignant solid tumor of the abdomen in children, accounting for >90% of all malignant renal pediatric tumors; improvement of treatment approaches has led to an increase in the overall survival rate to 85% (17). However, due to tumor metastasis and recurrence, and the development of resistance to chemoradiotherapy, some cases remain incurable (18,19). Therefore, it is crucial to identify novel key biomarkers for nephroblastoma, evaluate the risk of metastasis in a timely manner, accurately predict patient survival and improve the overall survival rate through targeted precision therapies.

NUSAP1 is an essential microtubule-binding protein in proliferating cells. Abnormal mitotic process during the cell cycle is one of the most significant causes for the development of malignant tumors (20). NUSAP1 plays a significant role in mitotic spindle assembly, chromosome segregation and in the regulation of cell division (21). It has been reported that NUSAP1 is highly expressed in cancer. A previous study demonstrated that NUSAP1 was associated with poor prognosis in breast cancer, while it was considered as a potential biomarker for ductal carcinoma *in situ* (22). Based on database analysis, NUSAP1 expression was significantly upregulated in bladder cancer stem cells, and NUSAP1 could be used as a therapeutic target to inhibit the characteristics of bladder cancer stem cells (23). The abnormal expression of NUSAP1 in ovarian cancer has good predictive value for the occurrence and prognosis of ovarian cancer, and can be used as a candidate target for the diagnosis and treatment of OC (24). In addition, targeted NUSAP1 knockdown could attenuate the growth and metastasis of bladder cancer cells (25). However, the specific role and mechanism of NUSAP1 in nephroblastoma have not been previously reported. The results of the present study demonstrated that the expression of NUSAP1 was significantly increased in nephroblastoma cell lines. This finding was consistent with the results reported by Zheng *et al* (15). To further evaluate the effect of NUSAP1 on the proliferative, invasive and migratory abilities of nephroblastoma cells, the shRNA technology was used to knock down the expression of NUSAP4. The results showed that NUSAP1 silencing significantly reduced cell viability and proliferation, as well as the invasive and migratory abilities of nephroblastoma cells. These results indicated that, inhibiting the expression of NUSAP1 could significantly attenuate the malignant progression of nephroblastoma and NUSAP1 can be used as a prognostic marker for nephroblastoma.

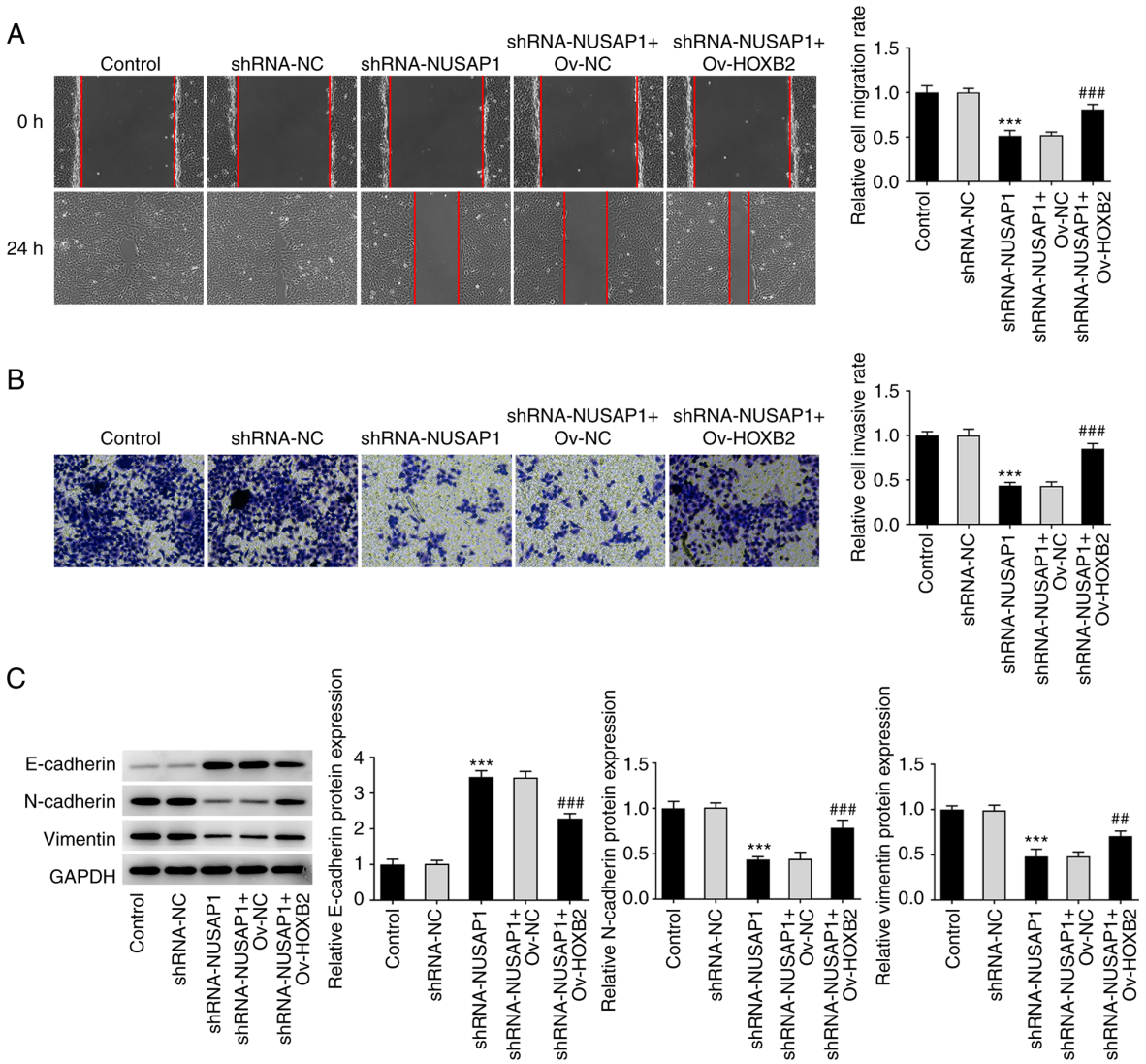

Figure 5. Overexpression of HOXB2 reverses the inhibitory effect of NUSAP1 knockdown on the metastasis of nephroblastoma cells. (A) Wound healing assay was performed to detect cell migration. (B) Transwell assay was conducted to detect cell invasion. Magnification, x200. (C) Western blotting was performed to detect the expression of metastasis-related proteins. ***P<0.001 vs. shRNA-NC; $^{\#\#}$P<0.01, $^{\#\#\#}$P<0.001 vs. shRNA-NUSAP1 + Ov-NC. HOXB2, homeobox protein Hox-B2; NUSAP1, nucleolar and spindle-associated protein 1; Ov, overexpression vector; shRNA, short hairpin RNA; NC, negative control.

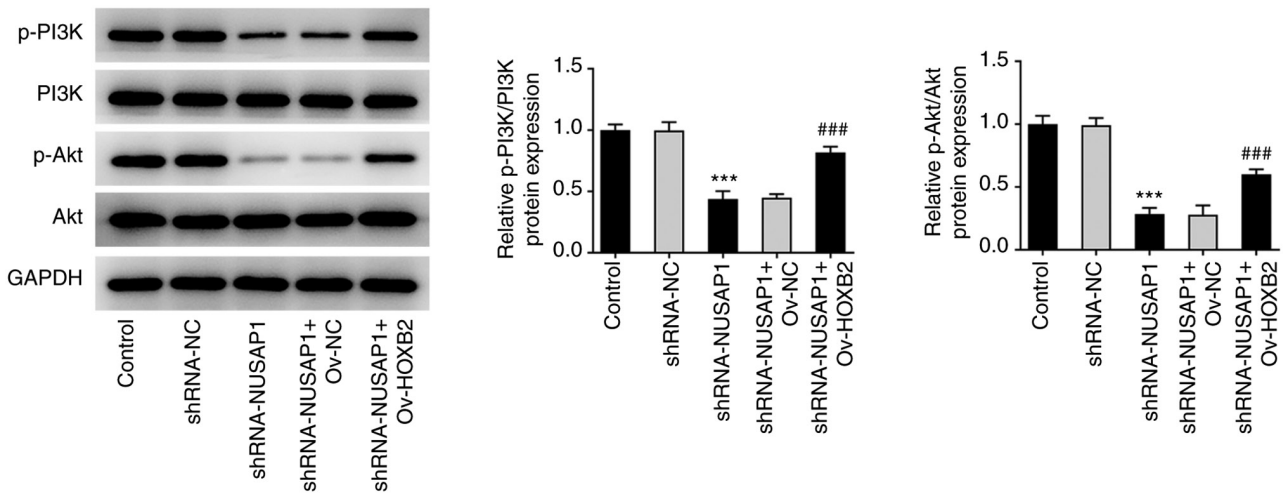

Figure 6. Overexpression of HOXB2 reverses the inhibitory effect of NUSAP1 knockdown on the proliferation and metastatic potential of nephroblastoma cells via the PI3K/Akt signaling pathway. Western blotting was performed to detect the PI3K/Akt-related proteins. ***P<0.001 vs. shRNA-NC; $^{\#\#\#}$P<0.001 vs. shRNA-NUSAP1 + Ov-NC. HOXB2, homeobox protein Hox-B2; NUSAP1, nucleolar and spindle-associated protein 1; Ov, overexpression vector; shRNA, short hairpin RNA; NC, negative control; p-, phosphorylated.

The binding capacity of TF HOXB2 on the NUSAP1 promoter was predicted using the JASPAR website. However, the effects of HOXB2 and NUSAP1 on nephroblastoma have not been previously investigated. In the present study, the regulatory effect of HOXB2 on NUSAP1 was verified via dual-luciferase reporter and ChIP assays. It has been reported that HOXB2 serves a regulatory role in tumor development, progression and cell proliferation via acting as a TF to regulate the expression of downstream factors (12). A previous *in vivo* study on the functional screening of tumor progression regulators revealed that HOXB2 could regulate the development of breast cancer (26). Another study demonstrated that HOXB2 promoted the invasion of HOP-62 non-small cell lung cancer (NSCLC) cells via transcriptionally regulating the expression of metastasis-related genes (27). In addition, HOXB2 promoted glial cell proliferation and invasion *in vitro* in 1,001 patients with glioma, and it was positively correlated with tumor grade and established as an independent prognostic biomarker (28). However, the effects of HOXB2 and NUSAP1 on the proliferation, invasion and migration of nephroblastoma cells remain unclear. The current study demonstrated that HOXB2 overexpression reversed the inhibitory effect of NUSAP1 knockdown on the proliferation and metastasis of nephroblastoma cells.

In the present study, the activation of the PI3K/Akt signaling pathway was inhibited following NUSAP1 silencing in nephroblastoma cells. However, this effect was reversed after HOXB2 overexpression. Therefore, it was hypothesized that HOXB2 overexpression could abrogate the inhibitory effect of NUSAP1 knockdown on the proliferation and metastasis of nephroblastoma cells via the PI3K/Akt signaling pathway. Consistent with the aforementioned findings, Li *et al* (29) showed that the expression levels of p-PI3K and p-Akt were decreased in glioblastoma cells following HOXB2 knockout. These results suggested that HOXB2 could promote the activation of PI3K/Akt signaling. In addition, NUSAP1 knockdown could also inhibit the activation of B cell translocation gene 2/PI3K/Akt signaling, thus attenuating cell proliferation and metastasis in NSCLC (2,30). This finding was consistent with the results of the current study.

However, there were certain limitations of the present study. First, the mechanism underlying the involvement of the PI3K/Akt signaling pathway was not further investigated. Second, only *in vitro* experiments in nephroblastoma cell lines were performed. Therefore, *in vivo* experiments are needed to further verify the results of the present study. These experiments will be performed in our future studies. In addition, we will further detect the influence of HOXB2/NUSAP1 on cell resistance caused by cisplatin or radiotherapy drugs in the next experiment.

In conclusion, the findings of the present study demonstrated that the TF HOXB2 could upregulate NUSAP1 to promote the proliferation, invasion and migration of nephroblastoma cells via the PI3K/Akt signaling pathway, thereby possibly providing novel targets for the diagnosis and treatment of nephroblastoma.

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Availability of data and materials

The datasets generated and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

BL and SF wrote the manuscript and analyzed the data. TL, JW, ZQ, YZ and BH performed the experiments and supervised the study. ZQ, YZ and BH searched the literature and revised the manuscript for important intellectual content. BL and SF confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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