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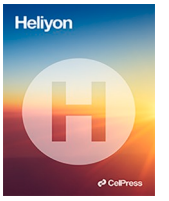

Research article

HOXB5 promotes the progression and metastasis of osteosarcoma cells by activating the JAK2/STAT3 signalling pathway

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ABSTRACT

Objective: To investigate the involvement of the homeobox gene B5 (HOXB5) in the progression and metastasis of osteosarcoma.
Methods: The expression of HOXB5 in human osteosarcoma tissues and its correlation with clinical indicators were investigated using bioinformatics analysis and immunohistochemical labelling. Human osteosarcoma cells (HOS, MG63, U2OS, and Saos-2) and normal human osteoblasts (hFOB1.19) were cultivated. The expression of HOXB5 in these cells was detected using western blotting (WB) and RT−PCR. Two cell lines exhibiting elevated HOXB5 expression were chosen and divided into three groups: the blank group (mock), control group (control) and transfection group (shHOXB5). The transfection group was infected with lentivirus expressing shRNAs targeting HOXB5. The transfection efficiency was detected by WB. Cell proliferation suppression was measured by CCK-8 and 5-ethynyl-2′-deoxyuridine (EdU) assays; the percentage of apoptotic cells was determined by flow cytometry; and cell migration and invasion were detected via the Transwell chamber test. WB was utilized to determine the protein expression of genes linked to metastasis (MMP2, MMP9), apoptosis (Bax, Bcl-2), and the JAK2/STAT3 pathway (JAK2, *p*-JAK2, STAT3, p-STAT3).
Results: In osteosarcoma tissues, HOXB5 expression was elevated and strongly correlated with distant metastasis. Silencing HOXB5 reduced the proliferation, migration and invasion of osteosarcoma cells; prevented the progression and metastasis of tumours in tumour-bearing nude mice; and reduced the activation of key proteins in the JAK2/STAT3 signalling pathway.
Conclusion: Through the JAK2/STAT3 signalling pathway, HOXB5 plays a crucial role in the malignant progression of osteosarcoma and is a promising target for osteosarcoma treatment.

1. Introduction

Osteosarcoma (OS), which usually originates from malignant mesenchymal cells of bone, is one of the most common aggressive bone tumours [1]. The majority of occurrences happen in children and adolescents, and the peak frequency is during the teenage

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growth spurt [2]. Despite the fact that OS detection and systemic treatment have gotten better recently, if lung metastasis occurs, the 5-year survival rate drops sharply to 20 % [3,4]. Thus, identifying new targets and explaining the molecular underpinnings of recurrence and metastasis are crucial for newer OS therapy options [5].

The homeobox (HOX) superfamily encodes transcription factors that play important roles in the progression of different kinds of malignancies [6]. The human HOX family comprises 39 genes located on four different chromosomes (clusters HOXA, HOXB, HOXC and HOXD). Moreover, the protein products of these genes have been shown to operate as transcriptional activators or suppressors during cancer formation [7]. In particular, HOXB5, a well-known HOX family member, has been shown to have an oncogenic role in the genesis and progression of various cancer types, including hepatocellular carcinoma (HCC) and pancreatic, colorectal, and breast cancer [8–11]. Specifically, HOXB5 promotes HCC metastasis through transactivating fibroblast growth factor receptor 4 (FGFR4) and C-X-C motif chemokine ligand 1 (CXCL1) expression. Furthermore, upon HOXB5 overexpression, myeloid-derived suppressor cell (MDSC) infiltration via the CXCL1/CXCR2 axis increases. In addition, HOXB5 increases the synthesis of MMP proteins and activates the ERK1/2 pathway, which allows retinoblastoma cells to migrate [12]. In non-small cell lung cancer, the dysregulation of HOXB5 facilitates malignant progression by enhancing the Wnt/β-catenin pathway [13]. However, the specific role of HOXB5 in OS and the underlying mechanism have remained unclear until recently.

Recently, ample evidence has indicated that the Janus kinase 2/signal transducer activator of transcription 3 (JAK2/STAT3) signalling pathway is frequently aberrantly activated in various malignant tumours and is involved in tumorigenesis, invasion, and metastasis [14]. This pathway is utilized to transduce signals from the extracellular space to the nucleus upon the binding of cytokines and growth factors to specific cell surface receptor extracellular domains and plays a crucial role in the clinical progression of colorectal cancer [15], hepatocellular carcinoma [16] and osteosarcoma [17]. Notably, the role of the JAK2/STAT3 signalling pathway in the malignant progression of OS mediated by HOXB5 has yet to be determined.

Using publicly accessible datasets, we examined differentially expressed genes (DEGs) between human OS tissues and normal tissues in the present study, and the results revealed that HOXB5 was upregulated in OS tissues. We investigated the expression of HOXB5 in OS tissues from surgical patients and found that HOXB5 was upregulated in OS tissue compared to adjacent normal tissue, which was supported by immunohistochemistry. Subsequent research into the role of HOXB5 silencing in OS cells demonstrated that inhibiting HOXB5 might prevent OS cell growth and metastasis both in vitro and in vivo. Furthermore, HOXB5 knockdown decreased JAK2 and STAT3 expression, while the STAT3 activator colivelin clearly reversed the tumour suppressor effects of HOXB5 knockdown on OS development overall. These findings suggested that HOXB5 is essential for tumorigenesis and metastasis through the JAK2/STAT3 signalling pathway and may be a marker for clinical diagnosis and a potential target of novel therapeutic strategies for osteosarcoma patients.

2. Materials and methods

2.1. Publicly available datasets and differentially expressed genes

GSE126209, GSE218035, and GSE16088 files were retrieved from the Gene Expression Omnibus database (GEO, https://www.ncbi.nlm.nih.gov/geo/), with the GSE126209 data containing 12 OS tissue and 11 nontumour tissue samples. GSE218035 was made up of 23 OS and matched normal tissue samples. GSE16088 comprised 14 OS tissue samples with clinical follow-up data. The "limma" tool in R software was used to identify DEGs between osteosarcoma and normal tissue, and adjusted P values 0.05 and |logfold change (FC)| >1 were utilized as significant criteria. The results are shown using the "ggplot2" and "pheatmap" tools to create volcanic maps and heatmaps.

2.2. Tissue samples and cell lines

In this study, tissue microarray (TMA) construction was performed using 29 osteosarcoma samples fixed in paraffin and 13 randomly chosen comparable nearby normal tissues that had undergone surgery without preoperative chemotherapy or radiotherapy between October 2015 and December 2022 from our research institution. Table 3 also provides a summary of the clinical and pathological information, such as age, sex, disease stage, and metastasis. HOS, MG63, U2OS, Saos-2 and the normal human osteoblastic cell line hFOB1.19 were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultured in DMEM (Gibco, USA) containing 10 % FBS and 1 % penicillin/streptomycin at 37 °C in an environment with 5 % CO2, except for hFOB1.19, which was cultured in DMEM/Ham's F-12 at 34 °C with 5 % CO2 supplemented with 10 % FBS and 400 mg/mL of geneticin. When the cells reached 80–90 % confluence, they were subcultured. The protocol of the current study was examined and granted clearance by the Clinical Medical Research Ethics Committee.

Table 1
| The Sequences of shRNA.

ShRNA1	5′-GCTTCACATCAGCCATGATAT-3′
ShRNA2	5′-CAGCGCCAATTTCACCGAAAT-3′
ShRNA3	5′-CGGCTACAATTACAATGGGAT-3′
ShNC	5′-TTCTCCGAACGTGTCACGT-3′

2.3. Immunohistochemistry analysis

Tumour tissues and adjacent nontumoral tissues were fixed in 10 % formalin and embedded in paraffin. Tissue sections of 5 μm were deparaffinized and dried, followed by incubation in sodium citrate buffer, then incubated for 30 min at room temperature in PBS containing 3 % hydrogen peroxide. The sections were incubated with primary antibodies (anti-HOXB5, 1:100, ThermoFisher) overnight at 4 °C, followed by reaction enhancing solution (PV-9000, ZSGB-BIO, China) at 37 °C for another 20 min. They were then incubated with the appropriate secondary antibodies for 35 min before being subjected to 3,3′-diaminobenzidine (DAB) staining and counterstaining by haematoxylin. After being dried in xylene, all tissue pieces were mounted using neutral glue. Using light microscopy, two pathologists unaware of the clinical data independently graded the tissue staining.

2.4. shRNA construction and transfection

The normal methodology was followed for transfecting Saos-2 and U2OS cells, and Genechem provided all of the shRNA (Shanghai, China). Using puromycin (Beyotime, China), the transfected cells were screened. Table 1 shows the HOXB5 shRNA sequences.

2.5. Cell viability assay

To test the proliferation effects of HOXB5, transfected Saos-2 and U2OS cells were seeded into a 96-well plate at 5×10^3 cells per well for 24 h at 37 °C. The culture was maintained for another 2 h, and the absorbance at 450 nm was measured at 0, 24, 48, 72, and 96 h using a BioTek microplate reader (Gene Co., Ltd., Shanghai, China) with 10 μL CCK-8 reagent (Dojindo, Japan).

2.6. 5-Ethynyl-2′-deoxyuridine (EdU) assay

For one day, osteosarcoma cells were grown on 6-well plates. After that, each well received 10 μM 5-ethynyl-2′-deoxyuridine (Beyotime, China), and it was incubated with the cells for 2 h at 37 °C. After permeabilizing the cells and fixing them with 4 % paraformaldehyde, the nuclei were stained with 500 μL of Hoechst 33342. An inverted fluorescence microscope was used to take photographs, and the proportion of EdU-positive cells was determined.

2.7. Wound healing assay

Six-well plates were seeded with 1×10^6 Saos-2 or U2OS cells. After the cells had attained 90 % saturation, they were scraped using a 200 μL plastic pipette tip to generate a gap in the cell coverage. After 48 h of culture in low-serum medium (1 %), cell migration rates were measured and compared for each group (the wound area after 48 h of culture divided by the wound area immediately after the scratch).

2.8. Transwell assay

The migration and invasion of osteosarcoma cells was evaluated by Transwell assays (8 μm pore, Corning, Inc.). 4×10^4 number of cells seeded for each group (Control, Mock and shHOXB5 group) into the top of the migration/invasion chambers, shRNA-transfected Saos-2 and U2OS cells were selected as the shHOXB5 group. The inner compartments of the Transwell inserts were filled with 200 μL of serum-free media containing 4×10^4 cells, while complete media with 10 % FBS was poured into the lower chambers. Unlike the migration experiment, the invasion assay necessitated the top chamber to be filled with 100 μL of diluted Matrigel (BD Biosciences, Sparks, USA) prior to the addition of cells. Following a 24-h incubation period, the inserts' bottom surface underwent two cold PBS washes, 4 % polyoxymethylene fixation, and 0.1 % crystal violet staining.

2.9. Western blotting

Osteosarcoma cells were lysed using RIPA (Epizyme, China) with PMSF (Epizyme, China), and the concentration of the protein lysates was measured using the BCA test. Proteins with different molecular masses were separated using SDS−PAGE, transferred to PVDF membranes, and after 1 h of blocking, they were incubated with the indicated primary antibodies overnight at 4 °C. The next day, the membrane was treated for 1 h with the relevant secondary antibodies, after which the stained bands were visualised with an ECL system and quantified with ImageJ. Primary antibodies against the following proteins were used: HOXB5 (1:1000, Abcam); Bcl-2, Bax, MMP2, MMP9 (1:1000, Affinity); JAK2, *p*-JAK2, STAT3, p-STAT3, β-actin (1:5000, Abmart).

2.10. Quantitative real-time polymerase chain reaction (qRT–PCR)

Using Total RNA Extraction Reagent (Vazyme Biotech, Nanjing, China), total RNA was isolated from the cells. HiScript II Reverse Transcriptase (Vazyme, Nanjing, China) was then used to reverse transcribe the RNA into cDNA in accordance with the manufacturer's instructions. The QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher Scientific, USA) was used to perform qPCR. Table 2 shows a list of the primer sequences. We determined the relative quantification of mRNA expression using the $2^{-\Delta\Delta Ct}$ approach.

2.11. Annexin V-APC/PI double staining

Following cell collection and rinsing in cold PBS, 5 μL of Annexin V-APC and 5 μL of PI (Bestbio, China) were incubated for 10 min and 3 min, respectively, at 4 °C without light. Flow cytometry (FCM, Beckman, CytoFLEX S) was used to determine the proportion of cells that underwent apoptosis.

2.12. H&E staining

Paraffin-embedded lung tissue samples from the nude mice were cut into 4 μm thick sections, deparaffinized with xylene, and rehydrated through graded alcohol washes, followed by staining with haematoxylin and eosin in steps. Subsequently, dehydration with different concentrations of alcohol was performed again. After this, the slides were dehydrated with xylene and sealed with neutral gum.

2.13. Animal experiments

Animal Experimental Committee granted permission, and all animal research were carried out in accordance with the Experimental Animal Ethics Committee's requirements. Skbex Biotechnology Co., Ltd. (Henan, China) provided a total of five-week-old female BALB/c nude mice, which were raised under specific pathogen-free (SPF) conditions until six weeks of age. For establishment of the xenograft mouse model, the nude mice were randomized into two groups and 1×10^6 shHOXB5 or shNC Saos-2 cells were suspended in 200 μL of cold PBS, which was then subcutaneously injected into the mouse's dorsolateral region. Every three days, the tumour's length (L) and breadth (W) were measured, and the volume was recorded using the following formula: volume of tumour $= 0.5 \times L \times W^2$. Eventually, all mice were sacrificed after 30 days and promptly dissected to measure the tumour weights. To construct the metastatic model, 1×10^6 shHOXB5 or shNC Saos-2 cells were injected into nude mice via the tail vein in 200 μL PBS. The mice were sacrificed 30 days later, and H&E staining was applied to the lungs.

2.14. Statistical analysis

GraphPad Prism 8.0.1 was used for all testing. Statistical significance of normally distributed data provided as the mean ± SD was determined using the two-sided Student's *t*-test, paired *t*-test, or one-way ANOVA as appropriate. The relationship between HOXB5 protein levels and clinicopathological traits was investigated using the chi-square test. $P < 0.05$ was designated as the level of statistical significance.

3. Results

3.1. In patients with osteosarcoma, HOXB5 expression is elevated and positively associated with metastasis

We first used the GEO database (GSE126209, GSE218035, and GSE16088) to identify promising treatment targets for OS. The findings showed that OS tissues had significantly greater HOXB5 mRNA expression than normal tissues (Fig. 1A and B).

TARGET datasets were used to assess HOXB5 expression to confirm its involvement in OS, and the results were consistent with those described above (Fig. 1C). At the same time, we further used the TARGET database to download the survival data of 87 patients with osteosarcoma, including 65 patients without metastasis and 22 patients with metastasis. After combining with the expression data, prognostic analysis and correlation analysis of metastasis were performed. The results showed that patients with high HOXB5 expression not only had worse prognosis than those with low expression (Fig. 1D), but also high HOXB5 expression was associated with metastasis in patients with OS (Fig. 1E). In addition, IHC was used to investigate HOXB5 expression in 29 OS samples and 13 neighbouring normal tissues. Fig. 1F shows representative images of cells with various HOXB5 expression levels. Remarkably, 61.5 % (8/13) of the adjacent normal tissues had low HOXB5 expression, but 72.4 % (21/29) of the OS tissues had significant HOXB5 protein expression (Fig. 1G). We examined the correlation between HOXB5 expression and clinicopathological characteristics to ascertain the clinical importance of HOXB5 in OS. As revealed in Table 3, increased HOXB5 expression was significantly associated with distant metastasis ($P = 0.044$), which was consistent with our findings.

3.2. HOXB5 promotes the proliferation of osteosarcoma cells

HOXB5 expression in OS cell lines (HOS, MG63, Saos-2, and U2OS) was assessed by qPCR and western blotting to further explore its

Table 2
The sequences of the mRNA primers.

HOXB5_F	5′-AACTCCTTCTCGGGGCGTTAT-3′
HOXB5_R	5′-CATCCCATTGTAATTGTAGCCGT-3′
GAPDH_F	5′-CGGCTACAATTACAATGGGAT-3′
GAPDH_R	5′-TTCTCCGAACGTGTCACGT-3′

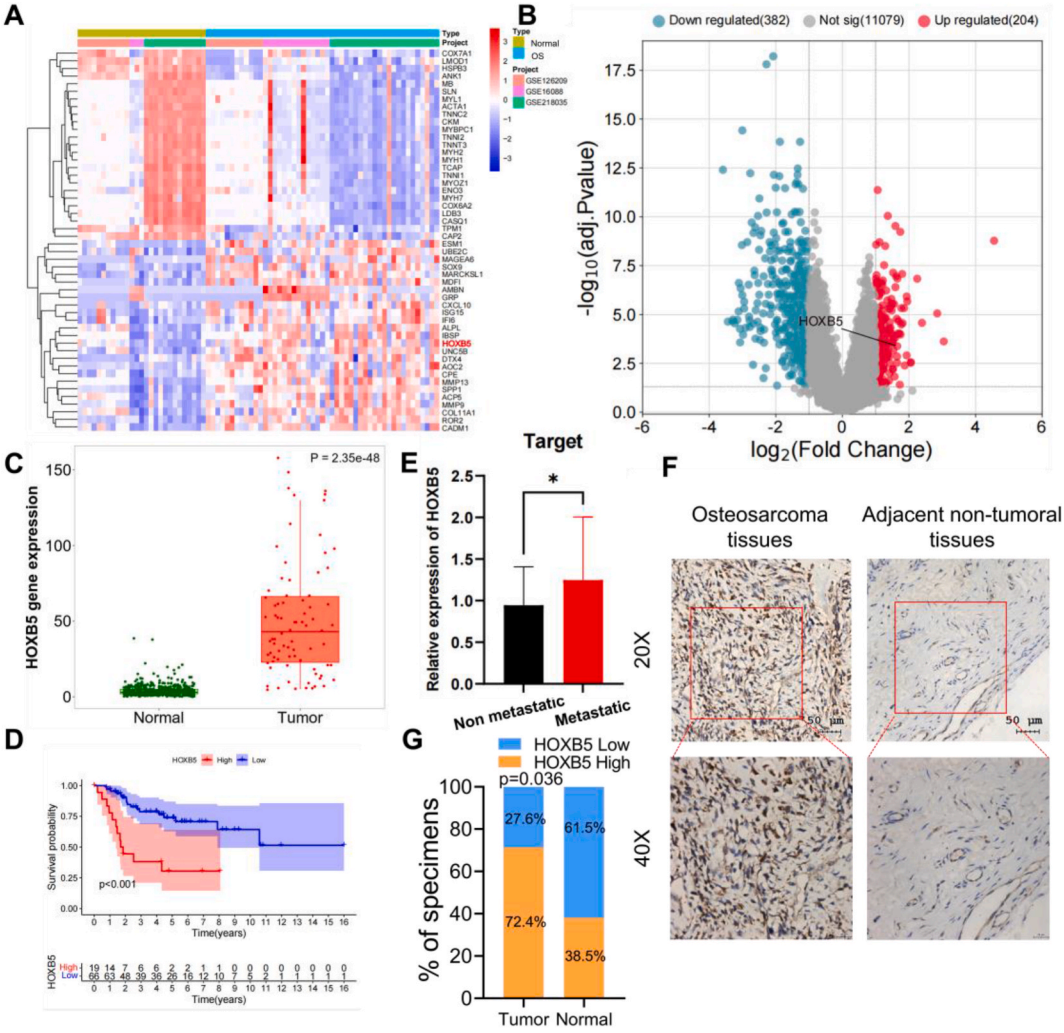

Fig. 1. The expression of HOXB5 is elevated in osteosarcoma tissues. (A) DEG heatmap comparing the OS and normal groups. (B) DEGs between the normal and OS groups shown in a volcano plot. (C) HOXB5 mRNA expression in OS patients was assessed using data from the TARGET database. (D) Kaplan-Meier survival analyses of HOXB5 expression in OS patients from the TARGET datasets. (E) The TARGET dataset showed that high HOXB5 expression was associated with metastasis in patients with OS. (F) Immunohistochemical evaluation of HOXB5 expression in OS tissues. (G) Statistical analysis of HOXB5 expression in OS and nearby normal tissues. The final score was calculated by multiplying the stained area by the intensity. A final score of 0–4 indicates poor expression and a score of ≥5 indicates high expression.

function in the pathogenesis of OS in vitro. The findings showed that Saos-2 and U2OS cells, which were used in the subsequent assays, had the highest abundance of HOXB5 (Fig. 2A–C). Owing to the high expression of HOXB5 in Saos-2 and U2OS cells, three shRNAs were used to silence HOXB5 (shHOXB5 group), and a negative control group was generated from OS cells; parental OS cells harbouring no lentivirus served as the mock group. Among the three shHOXB5 shRNAs, shRNA-3 exhibited the greatest silencing efficiency in SaoS-2 cells (Fig. 2D and E), whereas in U2OS cells, shRNA-1 had the strongest silencing impact (Fig. 2G and H) as detected by western blotting for HOXB5 expression. Thus, we selected shRNA-1-transfected Saos-2 cells and shRNA-3-transfected U2OS cells as the shHOXB5 group of corresponding OS cell lines for further study. Subsequently, the CCK-8 assays demonstrated that the capacity of the shHOXB5 group to proliferate was noticeably lower than that of the other two groups (Fig. 2F and I). Moreover, the EdU test was used to evaluate cell proliferation, and the findings were equivalent (Fig. 2L and M). The percentage of EdU-positive Saos-2 and U2OS cells in the total number of cells were shown in Fig. 2J and K. These data imply that HOXB5 may increase OS cell growth in vitro.

3.3. Osteosarcoma cell invasion and migration are facilitated by HOXB5

Wound healing and Transwell assays were also conducted to better understand the role of HOXB5 in the aetiology of OS. According to the wound healing assay, compared to that in the control and mock groups, silencing HOXB5 resulted in reduced cell migration (Fig. 3A–D). Similarly, in the Transwell assays (including migration and invasion assays), when the shHOXB5 group was compared to

Table 3
The correlation between HOXB5 expression and clinical pathology Parameters for OS.

Parameters	Case (n = 42)	Expression		p value
		High (n = 26)	Low (n = 16)	
Age				0.890
≥24	7	4	3	
<24	35	19	16	
Gender				0.495
Male	12	5	7	
Female	30	16	14	
Tumour size				0.474
≥3 cm	18	11	7	
<3 cm	24	12	12	
Tumour site				0.628
Femur	23	11	12	
Tibia	13	8	5	
Other	6	4	2	
Enneking stage				0.760
I	15	7	8	
II	2	12	9	
III	6	4	2	
Metastasis				0.044*
Yes	12	10	2	
No	30	13	17	
Recurrence				0.470
Yes	11	5	6	
No	31	18	13	

*P < 0.05.

the control and mock groups, the percentage of cells that migrated or invaded across the membrane was noticeably lower (Fig. 3E–H).

In order to further explore the possible mechanism of HOXB5-mediated enhanced migration and invasion of OS cells, we performed KEGG pathway analysis of DEGs between the normal and OS groups. Surprisingly, JAK-STAT signalling pathway, apoptosis, and ECM-receptor interaction were closely correlated with cell phenotypes in this study (Fig. 4A). Among them, the ECM-receptor interaction pathway was closely related to tumour invasion and metastasis. Therefore, we downloaded the transcriptome data of OS from the Target database, organized the data into expression matrix using perl, and used GSEA software version 4.3.1 to perform single-gene GSEA enrichment analysis according to the median HOXB5 expression. A total of 52 genes were found to be enriched in ECM-receptor interaction, and the expression of these 52 genes was further revealed by volcano plot. As shown in Fig. 4B, the MMP9 was significantly overexpressed. Matrix metalloproteinases (MMPs) are a class of enzymes that promote tumour cell metastasis, and the markers MMP-2 and MMP-9 are frequently employed to detect tumour spread. The shHOXB5 group exhibited significant inhibition of MMP-2 and MMP-9 expression compared to the control and mock groups (Fig. 3I-L). We concluded that HOXB5 might promote OS cell invasion and migration in vitro based on these findings.

3.4. HOXB5 suppresses cell apoptosis in OS

Both KEGG pathway enrichment in DEGs and HOXB5 single gene GESA enrichment analysis based on Target database showed that apoptosis was significantly enriched (Fig. 4A and C), thus, the impact of HOXB5 on apoptosis in Saos-2 and U2OS cells was investigated via flow cytometry. The findings demonstrated that, compared to those in the control and mock groups, the proportion of apoptotic cells in the shHOXB5 group was greater (Fig. 4D–G). In addition, in contrast to the control and mock groups, the shHOXB5 group exhibited upregulation of the apoptosis marker Bax expression and suppression of the anti-apoptotic protein Bcl-2 expression (Fig. 4H–K). These experiments demonstrated that HOXB5 may prevent OS cells from undergoing apoptosis in vitro.

3.5. In vivo, osteosarcoma development and metastasis are inhibited by silencing HOXB5

To elucidate the function of HOXB5 in vivo in OS, nude mice were randomly divided into shHOXB5 and control groups and were subcutaneously injected with stably transfected Saos-2 cells to establish a human OS xenograft model. The tumour volume was recorded every 3 days, and at 30 days, all mice were euthanized. The shHOXB5 group exhibited a smaller tumour volume and lower tumour weight than did the control group (Fig. 5A–D). In addition, in the metastasis model, metastatic lesions were observed on the lungs of the control nude mice (Fig. 5E and F). Significant pulmonary metastasis was established in the control group by H&E staining of the lungs (Fig. 5G), while the shHOXB5 group displayed significantly fewer pulmonary metastatic lesions (Fig. 5H). And the quantitative statistics of metastasis were shown in Fig. 5K. At the same time, we further checked EMT markers such as MMP9 in lung metastases, and immunofluorescence results showed an increase in red fluorescence in lung metastases in the control group compared with the shHOXB5 group (Fig. 5I and J). Our findings suggested that suppressing HOXB5 might decrease OS malignant progression in vivo.

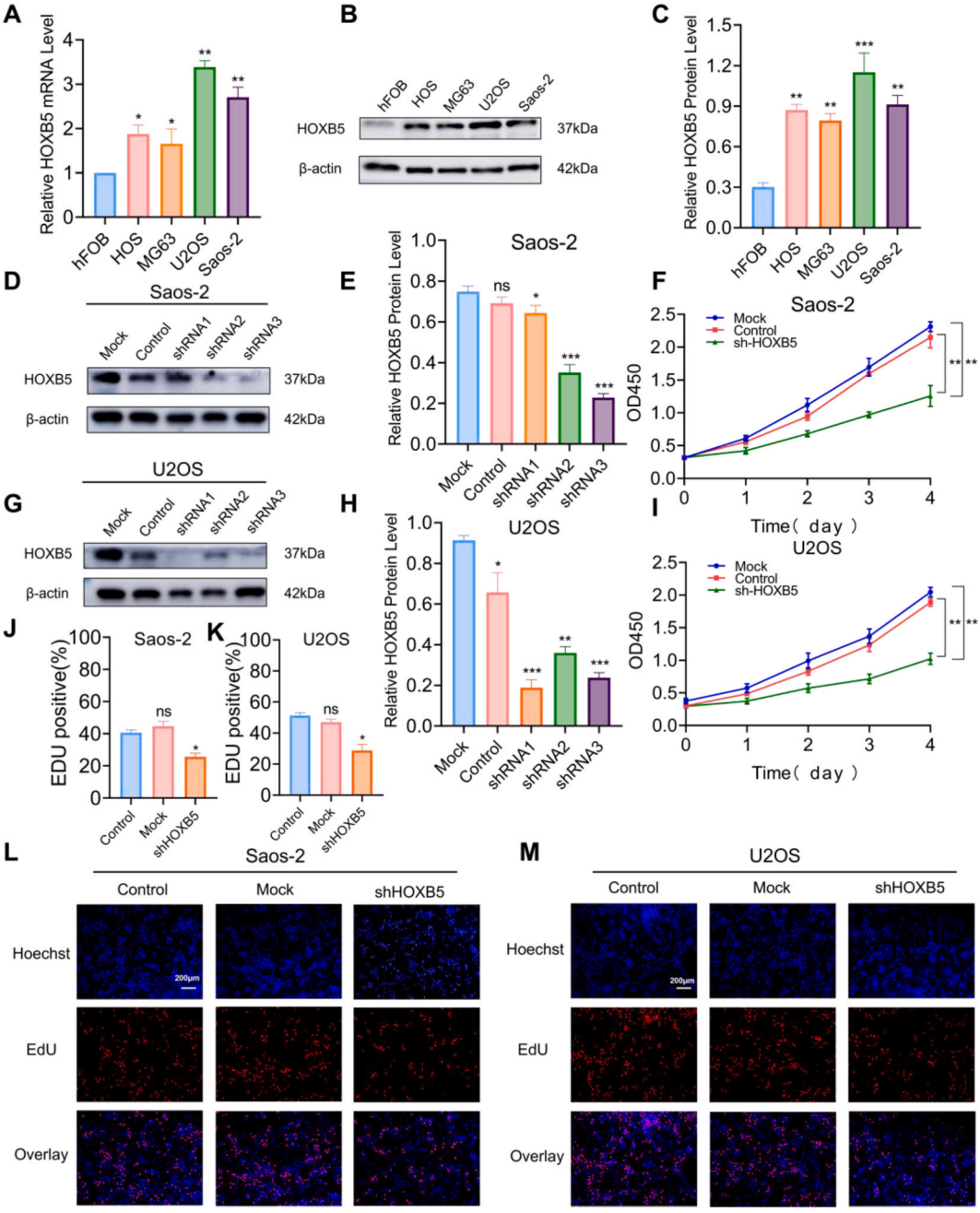

Fig. 2. Osteosarcoma cell growth is promoted by HOXB5. (A, B, C) qRT−PCR and WB were used to analyse the quantitative expression of HOXB5 in osteosarcoma cells and hFOB, respectively. (D, E, G, H) The efficiency of HOXB5 knockdown in Saos-2 and U2OS cells was quantified. (F, I) CCK-8 assays were used to assess the effect of HOXB5 silencing on proliferation. (J, K) Percentage of EdU-positive Saos-2 and U2OS cells to the total number of cells presented as means ± SD from 3 independent experiments. (L, M) EdU assays were used to quantify the impact of HOXB5 silencing on SaoS-2 and U2OS cell proliferation. ns, no significance. *P < 0.05, **P < 0.01, ***P < 0.001.

3.6. *HOXB5 promotes the progression of OS via the JAK2/STAT3 signalling pathway*

The malignant progression of OS, especially metastasis, is closely associated with the dysregulation of the JAK2/STAT3 signalling pathway [18,19]. Considering that high HOXB5 expression is closely related to distant metastasis, we further investigated whether silencing HOXB5 in Saos-2 and U2OS cells affects the JAK2/STAT3 signalling pathway. According to the western blotting results, *p*-JAK2 and p-STAT3, two important molecules in this pathway, were not phosphorylated when HOXB5 was silenced, which may explain the decreased proliferation and metastatic capacity of OS cells (Fig. 5L-O).

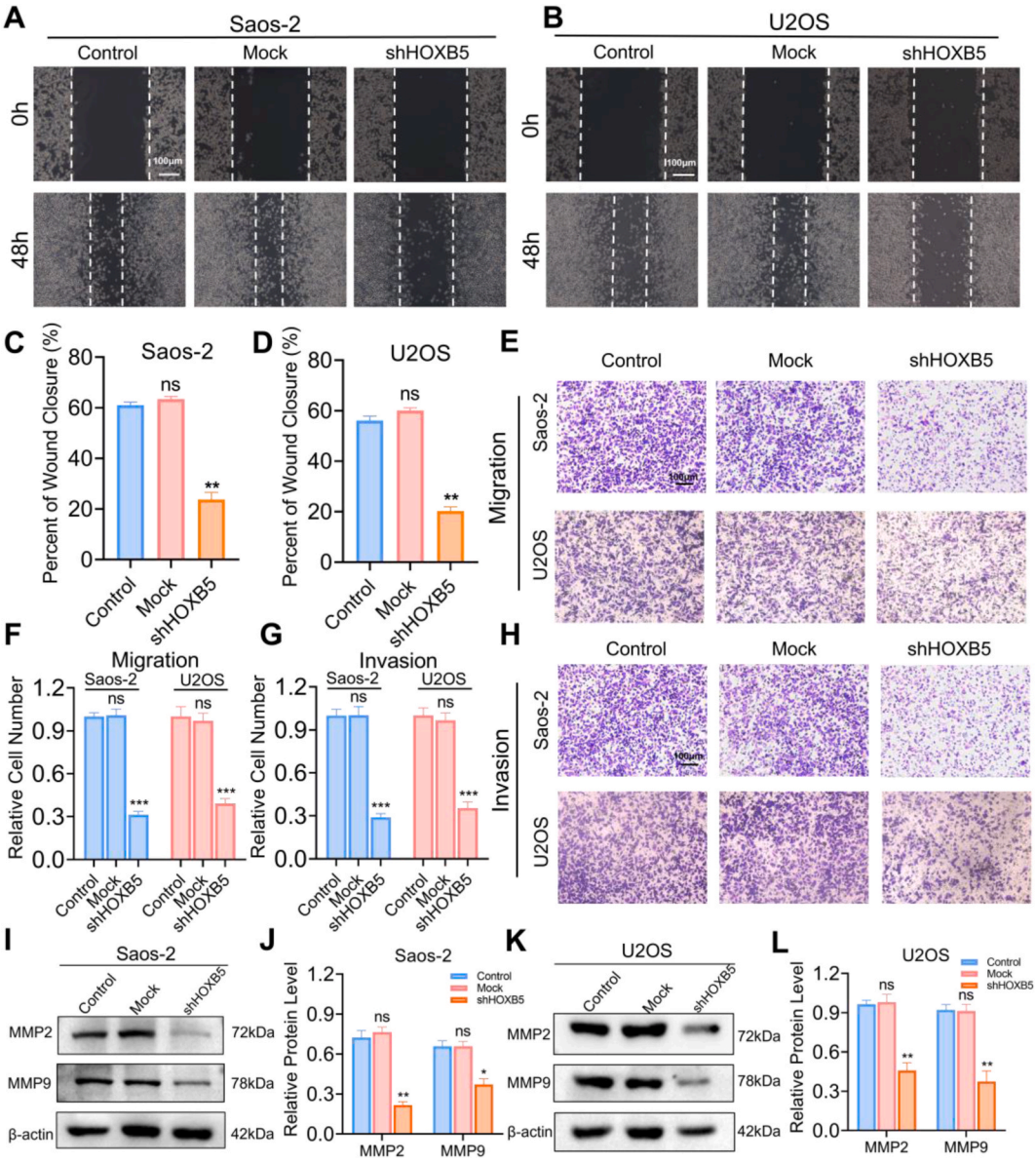

Fig. 3. Osteosarcoma cell invasion and metastasis are facilitated by HOXB5. (A, C) Quantitative examination of wound healing experiments revealed the migratory capacity of Saos-2 cells. (B, D) Quantitative examination of wound healing experiments revealed the migratory capacity of U2OS cells. (E, F) Quantitative analysis of Transwell migration tests was used to assess the cell migration capacity of Saos-2 and U2OS cells. (G, H) Cell invasion ability was evaluated using Transwell invasion assays. (I–L) Western blot was used to detect the relative expression of the MMP2 and MMP9 proteins in Saos-2 and U2OS cells. ns, no significance. $^{**}P < 0.01$, $^{***}P < 0.001$.

To further substantiate the regulatory function of the JAK2/STAT3 signalling pathway in HOXB5-mediated growth and metastasis of OS cells, shHOXB5 OS cells were stimulated with the STAT3 signalling activator colivelin (Selleck, USA), which was dissolved in pure water. Colivelin (5 μM) promoted the proliferation of shHOXB5 Saos-2 and U2OS cells as shown by EdU (Fig. 6A and B) and CCK8 (Fig. 6E and H) assays, but the cell proliferation rate in the "shHOXB5+Colivelin" group was still lower than that in the "shControl" group. After colivelin treatment, the percentage of EdU-positive Saos-2 and U2OS cells in the total number of cells were shown in Fig. 6C and D. Transwell assays demonstrated that colivelin may increase cell migration and invasion through Matrigel in shHOXB5 Saos-2 (Fig. 6F and G) and shHOXB5 U2OS cells (Fig. 6I and J). Treatment with colivelin also increased the migration of shHOXB5 U2OS and Saos-2 cells, as determined by wound healing assays (Fig. 6K-M). After treating cells with the STAT3 signalling activator colivelin, Western Blots were used to analyse its effect on the activation of JAK2/STAT3 signalling pathway (Fig. 6N-Q). Taken together, our research demonstrated that HOXB5 may facilitate OS metastasis and development through in vivo and in vitro stimulation of the JAK2/STAT3 signalling pathway.

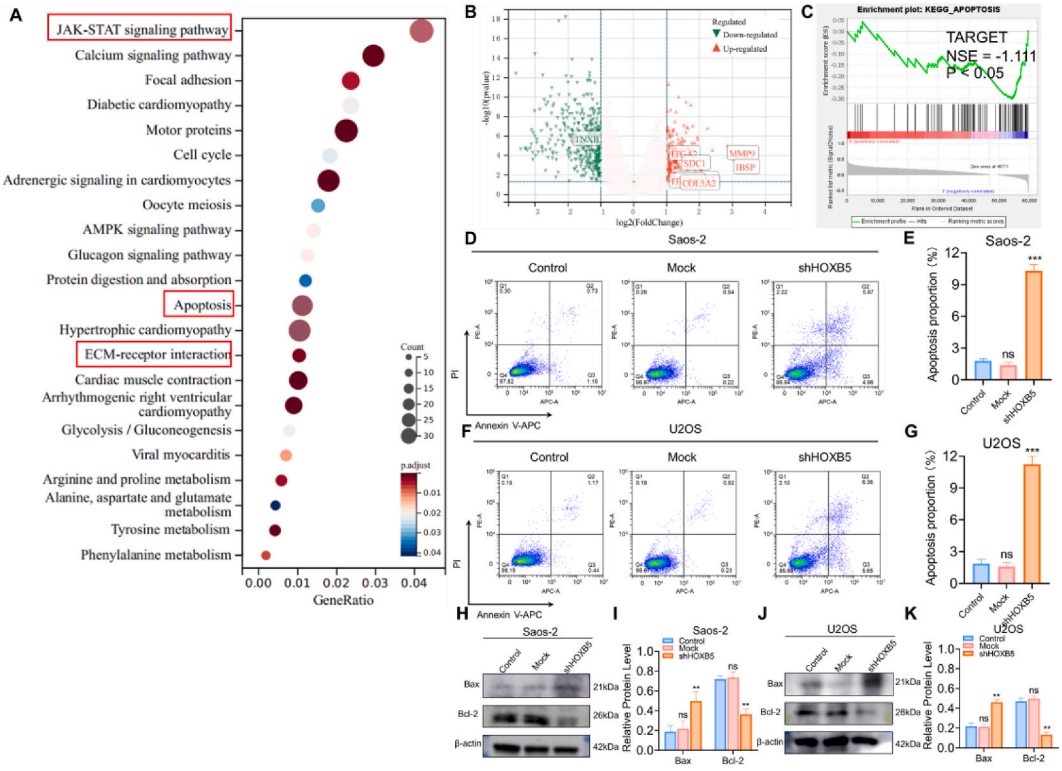

Fig. 4. Cell apoptosis is induced by HOXB5 knockdown. (A) KEGG pathway analysis of DEGs between normal and OS groups. (B) Volcano plot showed the enrichment of DEGs in the ECM receptor interaction pathway. (C) TARGET datasets showed that higher HOXB5 expression was associated with enrichment of apoptosis. (D–G) Utilizing flow cytometry, the impact of HOXB5 knockdown on SaoS-2 and U2OS cell apoptosis was identified and measured. (H–K) WB was used to detect the relative expression of apoptotic markers and proteins. ns, no significance. $^{**}P < 0.01$, $^{***}P < 0.001$.

4. Discussion

The importance of homeobox genes in Drosophila embryogenesis was initially demonstrated. It is interesting to note that *Drosophila melanogaster* mutants showed alterations in body growth and structure, such as the replacement of legs with antennae. These developmental abnormalities are caused by mutations in a phenomenon known as "homeotic" transformation. Additionally, throughout embryogenesis, the proteins encoded by homeobox genes function as essential master regulatory transcription factors [20]. In addition to interfering with angiogenesis, autophagy, proliferation, differentiation, and inflammation, they can also impact the expression of genes associated with stem cells and the activity of tumour-related metalloproteinases [21]. Consequently, it appears that both the up- and downregulation of transcription factors belonging to the HOX family, particularly the HOXB cluster, is crucial for initiating carcinogenesis. Zhou et al. discovered that HOXB1 activated the NF-kB pathway, which in turn increased the incidence of OS [22]. Li discovered that circ-0001785 regulates the pathogenesis of OS through upregulation of HOXB2 expression by sponge miR-1200 [23]. A recent study proposed that HOXB6 regulates the CC and CXC chemokines of the cytokine–cytokine receptor interaction signalling pathway, which likely causes the development of OS and preserves the characteristics of tumour stem cells [24]. As a member of the HOXB cluster in the homeobox (HOX) family, HOXB5 plays an immunosuppressive or carcinogenic role in many malignant tumours and it is closely related to a poor prognosis and a considerable possibility of metastases [25,26]. However, there are few studies on HOXB5 and musculoskeletal tumours, especially in OS with its high degree of malignancy. Thus, it would be highly beneficial to conducted more studies on the function of HOXB5 in OS.

OS exhibits a high degree of local invasiveness and a tendency for distant metastasis, which poses significant challenges to clinical treatment and results in a lack of effective therapeutic methods for advanced disease [5]. The process of epithelial–mesenchymal transition (EMT) is crucial for the development of secondary cancers [27]. The extracellular matrix (ECM) and basement membrane constitutes the first barrier in the process of tumour metastasis, and their degradation is one of the characteristic features of EMT. Matrix metalloproteinases (MMPs) show great potential in the degradation of the ECM. Once the activation of MMPs, which creates breaches in the barriers between tissues and enables cancer cells to enter and spread across nearby normal tissue, generating distant metastases, is a primary cause of this deterioration [28,29]. Surprisingly, in the present study, our findings revealed that HOXB5 knockdown could dramatically suppress OS cell migration and invasion as well as lower MMP2 and MMP9 expression. This suggests that HOXB5 influences the EMT process, which in turn encourages OS cell invasion and metastasis. HOX genes have previously been

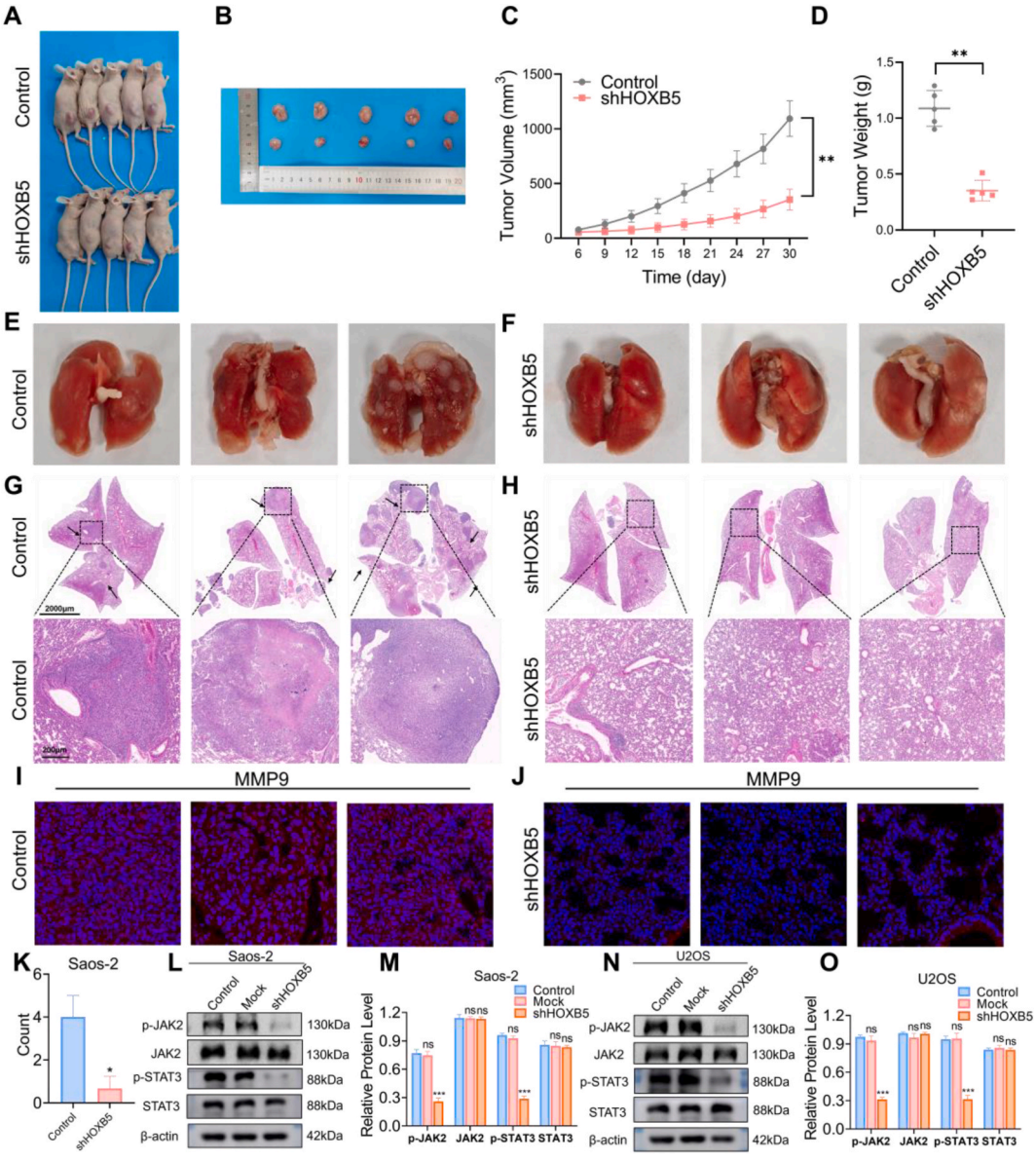

Fig. 5. In vivo osteosarcoma growth and metastasis are stimulated by HOXB5. (A) Representative images of the xenograft model. (B) Typical images of the corresponding groups of xenograft tumours. (C) Growth curves for tumours from the relevant categories. (D) The average weight of the tumours among the groups. (E, F) Typical images of lung metastases in nude mice. (G, H) H&E staining of lung metastases revealing histological characteristics. (I, J) The expression of EMT marker MMP9 in lung metastases by Immunofluorescence staining. (K) The quantification of the lung metastasis. (L–O) Western blot was used to detect the expression of *p*-JAK2, JAK2, p-STAT3 and STAT3 in Saos-2 and U2OS cells after silencing HOXB5.

found to be able to regulate the expression of MMPs during tumour progression. Recent studies have shown that HOXD11 activates the transcription of fibronectin 1 (FN1), leading to the degradation of the extracellular matrix through the FN1/MMP2/MMP9 pathway, promoting epithelial–mesenchymal transition in penile squamous cell carcinoma (PSCC) [30]. The growth and progression of tumours largely rely on angiogenesis. Anne et al. found that HOXB5 enhanced the migration of monocytes and endothelial cells in vitro and stimulated vascular remodelling by upregulating the inflammatory molecules monocyte chemotactic protein-1 (MCP-1) and interleukin-6 (IL-6) in a mouse hindlimb ischaemia model [31], which provided a certain theoretical support for the related research on HOXB5 in promoting tumour invasion and metastasis.

According to our findings, HOXB5 was found to promote the proliferation and metastasis of OS cells through the JAK2/STAT3 signalling pathway. Tyrosine kinase-related receptors, JAK2 and STAT3 proteins, which are involved in critical biological processes such haematopoiesis, immunological control, and developmental stages of embryos, make up the majority of this signalling pathway. Meanwhile, it has intimate connections with the proliferation, angiogenesis, and metastasis of malignancies in humans [32].

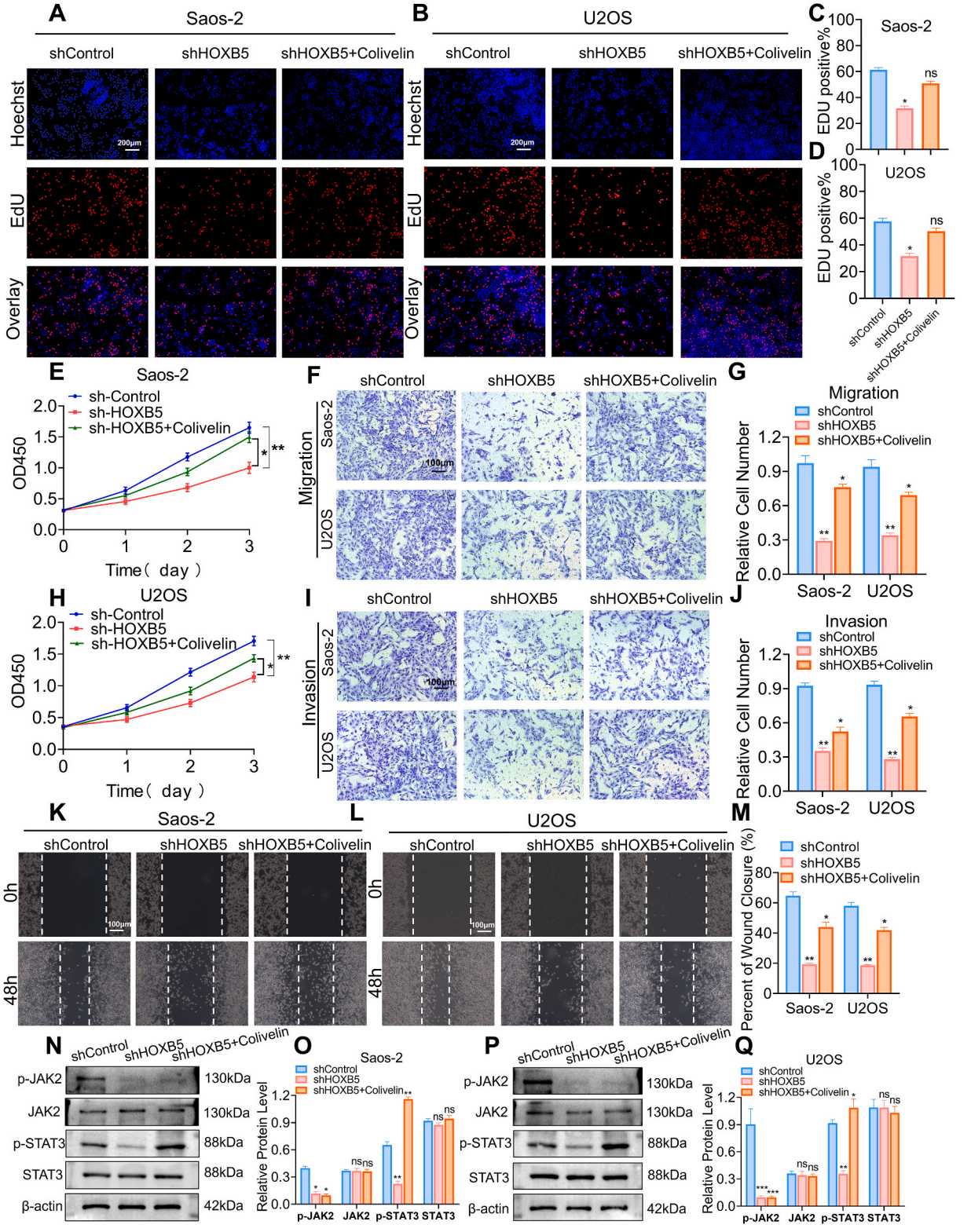

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Fig. 6. Induction of JAK2-STAT3 signalling mediated by colivelin. (A, B, E, H) An EdU assay and a CCK-8 assay were used to evaluate the effect of colivelin on the growth of shHOXB5 OS cells. (C, D) Percentage of EdU-positive Saos-2 and U2OS cells to the total number of cells. (F, G, I, J) Transwell migration and invasion assays were used to quantitatively analyse the effect of colivelin on the biological behaviour of shHOXB5 osteosarcoma cells. (K-M) The impact of colivelin on the migration of shHOXB5 OS cells was determined via wound healing experiments. (N-Q) Western blot and their quantitative analysis of treating colivelin on expression of *p*-JAK2, JAK2, p-STAT3 and STAT3 in Saos-2 cells. *P < 0.05, **P < 0.01, ***P < 0.001.

Angiogenesis in tumours and EMT are caused by this signalling pathway's malfunction, which also impairs cell differentiation and induces malignant transformation [33]. Normally, the JAK2/STAT3 pathway is activated when hormones like prolactin, growth factors like epidermal growth factor (EGF), and cytokines, primarily the interleukin-6 (IL-6) family, connect to the extracellular domain of particular cellular receptors (RTK). This causes JAK2 to phosphorylate each other on their tyrosine residues (known as autophosphorylation), activating their kinase domain. Then P-JAK2 phosphorylates STAT3, causing the STATs to dimerize, dissociate from the receptor, and translocate to the nucleus, where they bind to particular DNA sequences and trigger transcription of their target genes, such as cMyc, Bcl-2, P53, MMPs, and so on [34]. In recent years, Zhao et al. confirmed that HOXB5 promotes GSCs proliferation by activating IL6-mediated JAK2/STAT3 signalling [25]. Since HOXB5 is an important transcription factor, we hypothesized that prolactin and other hormones, epidermal growth factor (EGF) or cytokines may mediate the activation of JAK2/STAT3 signalling pathway by HOXB5 in OS, and we will conduct more detailed research in future work.

The JAK2/STAT3 pathway is also indispensable for bone homeostasis, and when it is disrupted, abnormal osteoblasts and osteoclasts are generated, which leads to skeletal disorders [35,36]. It has been demonstrated that the JAK2/STAT3 signalling pathway is frequently activated and overexpressed in OS, promoting its progression and metastasis [37,38], and it plays a role in radioresistance in colorectal cancer [15]. The chromatin immunoprecipitation (ChIP) study carried out by Lin et al. further validated a direct interaction between STAT3 and the EMT-related protein Slug [39]. The results of these previous studies demonstrated that JAK2/-STAT3 signalling promotes EMT and modulates tumour metastasis. Through the current investigation, we were able to determine that HOXB5 might regulate the production of proteins linked to EMT and exacerbate OS metastasis, since these effects were compromised by HOXB5 silencing. Moreover, a STAT3 activator (colivelin) partially reversed the tumour suppression of HOXB5 silencing, further suggesting that HOXB5 regulates OS cell proliferation and metastasis through the JAK2/STAT3 pathway.

Despite a great deal of research on the function of HOXB5 in OS, this study still has several limitations. To begin with, the downstream target genes in OS that HOXB5 directly controls remain unclear. Second, we found that the inhibited invasion and migration of shHOXB5 OS cells was partially, but not completely, reversible upon stimulating the JAK2/STAT3 signalling pathway. This finding suggests the involvement of other signalling pathways in HOXB5-mediated control of progress of OS. In addition, it is important to note that the differences in migration and invasion/metastasis might also be due, at least partially, to the effect on proliferation and might not only be ascribed to MMP9. This also objectively reflects the ability of HOXB5 to regulate tumour progression through multiple mechanisms, and subsequent studies should further explore other major molecules that may affect the proliferation or invasion/migration of OS cells. Last, but not insignificantly, in our metastasis model, the primary site of metastasis was the lungs rather than the liver, which is a unique phenomenon. In contrast to an earlier study, we did not investigate the processes behind this phenomenon, nevertheless, future studies will be focused on investigating the potential mechanisms.

In summary, our study demonstrated the overexpression of HOXB5 in OS and its association with metastasis in OS patients. HOXB5 promotes the progression and metastasis of OS cells through activation of the JAK2/STAT3 signalling pathway, indicating its potential as a target for gene therapy in OS.

Ethics declarations

This study was reviewed and approved by the Clinical Medical Research Ethics Committee of The First Affiliated Hospital of Anhui Medical University and Anhui Medical University's Animal Experimental Committee (Hefei, China), with the approval number: (PJ2022-10-15, LLSC20232175).

Informed consent was not required for this study because the tissue samples in this study are previous clinical surgical specimens from the Department of Pathology of the First Affiliated Hospital of Anhui Medical University. Moreover, we have applied to the Ethics Committee of the hospital for exemption from signing the informed consent and passed it.

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Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Qiming Ma: Writing – review & editing, Writing – original draft, Visualization, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Xingxing Li:** Writing – review & editing, Writing – original draft, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Huming Wang:** Writing – review & editing, Investigation, Formal analysis. **Shenglin Xu:** Validation, Supervision, Methodology, Formal analysis, Data curation. **Yukang Que:** Methodology, Formal analysis. **Peng He:** Methodology, Investigation. **Rui Yang:** Resources. **Qiwei Wang:** Supervision, Funding acquisition, Conceptualization. **Yong Hu:** Writing – review & editing, Supervision, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Qiwei Wang reports financial support was provided by Health research project of Anhui Province. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e30445.

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