

HOXB5-activated ANGPT2 promotes the proliferation, migration, invasion and angiogenic effect of esophageal cancer cells via activating ERK/AKT signaling pathway

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Abstract. Esophageal cancer, which is the eighth most common cancer worldwide, has a poor prognosis and high mortality rate. The present study was designed to investigate the proliferation, migration, invasion and angiogenic effect of the homeobox B5 (HOXB5)/angiopoietin-2 (ANGPT2) interplay in esophageal cancer. The relative expression of ANGPT2 and HOXB5 in esophageal cancer and the association between gene expression was evaluated using data from Gene Expression Profiling Interactive Analysis databases. Following transduction of short hairpin RNA-ANGPT2#1/2 plasmids, ANGPT2 was silenced. Viability, proliferation and invasion of esophageal cancer cells were assessed using CCK-8, 5-EdU, colony formation, wound healing and Transwell assays, respectively. Moreover, the transcriptional activity of ANGPT2 and angiogenesis were detected with luciferase reporter, chromatin immunoprecipitation (CH-IP) and tube formation assays. The results of the present study indicated that ANGPT2 was upregulated, both in esophageal cancer cell lines and tissue and there was an association between the ANGPT2 upregulation and the poor patient prognosis. In addition, ANGPT2 silencing suppressed esophageal cancer cell proliferation, migration, invasion and angiogenesis. The HOXB5 expression was also increased in esophageal cancer, and transcriptionally activated ANGPT2. Moreover, HOXB5 overexpression reversed the effects of ANGPT2 silencing in esophageal cancer cells. Furthermore, ANGPT2 silencing inactivated ERK/AKT signaling, whereas the HOXB5 overexpression blocked this effect. In conclusion, ANGPT2, which was transcriptionally activated by HOXB5, activated the ERK/AKT signaling pathway to promote

proliferation, metastasis and angiogenesis of esophageal cancer cells.

Introduction

As one of the most common types of gastrointestinal cancer, esophageal cancer has shown an increasing incidence in previous years (1,2). The detection rate of esophageal cancer has increased from 3.79% in 2016 to 5.42% in 2020 (3). Moreover, the overall survival rate of esophageal cancer is 15-25% (4). To date, several available treatments, including chemotherapy, radiotherapy, surgery and combined therapy, have improved the survival rate of esophageal cancer (5,6). Nevertheless, little improvement has been achieved in its mortality (7). To the best of our knowledge, the underlying mechanism and progression of esophageal cancer have not been fully determined.

Angiopoietin-2 (ANGPT2) is a growth factor that regulates vessel growth and maturation during angiogenesis (8,9). ANGPT2 expression is associated with tumor metastasis in numerous types of human cancer (10). For example, Urosevic *et al* (11) demonstrated that upregulation of ANGPT2 mediates liver metastasis in colon cancer. Moreover, ANGPT2 upregulation is associated with poor prognosis of patients with non-small cell lung cancer (12). However, the role of ANGPT2 in esophageal cancer development remains unclear.

Homeobox B5 (HOXB5), a member of the homeobox gene family, participates in the progression of multiple types of cancer, such as non-small cell lung (13) and gastric cancer (14) and head and neck squamous cell carcinoma (15). HOXB5 is reported to regulate a number of cancer cell functions, such as pancreatic, colorectal cancer, breast cancer and so on, and its overexpression is associated with cancer progression and poor patient prognosis (16-18). Nevertheless, the role of HOXB5 in esophageal cancer remains unclear; therefore, the present study aimed to investigate its underlying mechanism in the malignant progression of esophageal cancer.

Materials and methods

Cell culture and transfection. Human normal esophageal epithelial cell line (HEEC; cat. no. CP-H031) was obtained from Procell Life Science & Technology Co., Ltd. Human

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umbilical vein endothelial cells (HUVECs; cat. no. 3571773) and esophageal cancer cell lines, including KYSE-70 (squamous carcinoma cells; cat. no. ACC 363) and KYSE-30 (squamous carcinoma cells; cat. no. ACC 351), were obtained from BioVector NTCC Inc. and EC-9706 cell line (squamous carcinoma cells; cat. no. ZY6226) was purchased from Shanghai Zeye Biotechnology Co., Ltd. HEECs and HUVECs were immortalized cell lines. Dubelcco's modified eagle medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μ g/ml streptomycin was used to culture cells at 37°C with 5% CO₂.

To knock down ANGPT2 and upregulate HOXB5 expression in esophageal cancer cells, short hairpin RNA (shRNA) against ANGPT2 (sh-ANGPT2#1 and sh-ANGPT2#2; 50 nM), pcDNA3.1-HOXB5 (2 μ g), as well as corresponding negative control (shRNA-NC; 50 nM) and pcDNA3.1-NC (2 μ g) were obtained from Genscript Biotech Corporation. EC-9706 cells in logarithmic growth phase were inoculated into 6-well plates (6x10⁴ cells/well). EC-9706 cells at 90% confluence were transfected using Lipofectamine 2000[®] transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37°C. Following 48 h incubation, cells were collected for subsequent experiments. Sequence fragments that interfere with ANGPT2 are not be provided as the company did not provide them.

Reverse transcription-quantitative PCR (RT-qPCR). EC-9706 cells were placed in a 6-well plate (6x10⁴ cells/well). Following transfection, total RNA isolated from cells using TRIzol[®] reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol was reverse transcribed into complementary DNA using PrimeScript reverse transcriptase (Takara Biotechnology Co., Ltd.). Subsequently, qPCR was performed using SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) on the ABI PRISM 7900 System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR conditions: 95°C for 10 min for initial denaturation, 40 cycles of denaturation 15 sec at 95°C, annealing 30 sec at 60°C and elongation 30 sec at 72°C and final extension for 5 min at 72°C. Finally, the relative gene expression was calculated via the 2^{- $\Delta\Delta$ C_q} method (19) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) used as an endogenous control for HOXB5 and ANGPT2. The following primers were used for qPCR: HOXB5 forward, 5'-AACTCCTTCTCGGGCGT TAT-3' and reverse, 5'-CATCCCATTTGTAATTGTAGCC GT-3'; ANGPT2 forward, 5'-AACTTTCGGAAGAGCATG GAC-3' and reverse, 5'-CGAGTCATCGTATTCGAGCGG-3' and GAPDH forward, 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse 5'-GCGCCCAATACGACCAAATC-3'.

Western blot analysis. The extraction and quantification of total proteins from cells were conducted with RIPA lysis buffer (Beyotime Institute of Biotechnology) and BCA kit (Beyotime Institute of Biotechnology), respectively. After being separated by 10% SDS-PAGE, the proteins (30 μ g/lane) were then transferred onto PVDF membranes, as previously described (20). Membranes were blocked with 5% non-fat milk for 2 h at room temperature and then incubated with primary antibodies against ANGPT2 (1:1,000; cat. no. ab155106; Abcam), HOXB5 (1:1,000; cat. no. ab109375; Abcam),

E-cadherin (1:10,000; cat. no. ab40772; Abcam), N-cadherin (1:5,000; cat. no. ab76011; Abcam), Vimentin (1:1,000; cat. no. ab92547; Abcam), phosphorylated (p)-ERK (1:1,000; cat. no. ab201015; Abcam), p-AKT (1:1,000; cat. no. 9271; Cell Signaling Technology, Inc.), ERK (1:1,000; cat. no. ab17942; Abcam), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.) and GAPDH (1:10,000; cat. no. ab181602; Abcam) at 4°C overnight. Following primary antibody incubation, membranes were incubated for 2 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:20,000; cat. no. ab205718; Abcam). Finally, the protein signals were detected using enhanced chemiluminescence kit (Beyotime Institute of Biotechnology). ImageJ 1.50i software (National Institutes of Health) was used to analyze the blots. All results were verified using ≥ 3 independent experiments.

Cell Counting Kit (CCK)-8 assay. EC-9706 cells were inoculated into 96-well plates (1.5x10⁴ cells/well) and incubated for 24 h at 37°C. Subsequently, 10 μ l CCK-8 reagent (Beyotime Institute of Biotechnology) was added into each well and cells were incubated for another 3 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Inc.).

EdU staining assay. EC-9706 cells seeded into 6-well plates (6x10⁴ cells/well) were incubated at 37°C overnight. After exposure to 50 μ M EdU solution (Beyotime Institute of Biotechnology), the cells were incubated at 37°C for another 4 h. Then, the working solution was removed, followed by digestion with trypsin at 37°C for 3 min, centrifugation at 1,500 x g for 10 min at 4°C and fixation with 4% paraformaldehyde for 15 min at room temperature. Following permeation with 0.5% Triton X-100 at room temperature for 10 min, the cells were incubated with Click reaction solution in the dark for 30 min. The nuclei were counterstained for 15 min at room temperature with 100 ng/ml DAPI. Finally, the cells were observed under a fluorescence microscope (Olympus Corporation; magnification, x200).

Wound healing assay. EC-9706 cells were plated in 6-well plates (6x10⁴ cells/well) and cultured to 90% confluence in DMEM with 10% FBS at 37°C for 48 h. To make a straight scratch in the cell monolayer, a 200- μ l pipette tip was applied. After washing three times with PBS, the cells were then incubated in serum-free DMEM for 48 h at 37°C with 5% CO₂ and imaged at 0 and 48 h using a light microscope. The migration rate was calculated based on the formula: (Wound width at 0 h-wound width at 48 h)/wound width at 0 h x 100%. The images of the scratch areas were processed using ImageJ 1.50i software (National Institutes of Health).

Transwell assay. EC-9706 cells were inoculated into the upper chamber (6x10⁴ cells) containing serum-free DMEM of Transwell plates (EMD Millipore), which were precoated with Matrigel (37°C for 30 min) and incubated at 37°C for 24 h; complete medium with 10% FBS to the lower chamber of 6-well plates. After 24 h migration, the fixation and staining of EC-9706 cells was performed using 4% paraformaldehyde at room temperature and 0.1% crystal violet at room temperature

for 30 min each, respectively. The images of invasion were captured and the number of invading cells was counted using an inverted light microscope (Eclipse Ti2; Nikon Corporation).

Colony formation assay. EC-9706 cells were plated in 6-well plates (1×10^3 cells/well). After transfection, EC-9706 cells were continuously cultured for two weeks at 37°C in DMEM, which was replaced every 3 days. Then, 4% paraformaldehyde was used to fix the cell colonies for 20 min at room temperature, followed by staining using Giemsa (Beyotime Biotechnology Institute) for 20 min at a room temperature. The colonies containing >50 cells were imaged using a COOLPIX S520 digital camera (Nikon) and the number of clones was counted using Image J 1.50i software (National Institutes of Health).

Tube formation analysis in esophageal cancer cells. HUVEC cells (100 μ l) were seeded into a precooled 96-well plate (1.5×10^4 cells/well) before addition of 100 μ l/well Matrigel at 37°C for 30 min. Following incubation at 37°C for 24 h, the tube formation was monitored and imaged using an inverted light microscope (IX70; Olympus Corporation). Five visual fields were randomly selected and length of the lumen was calculated using Image Pro Plus (version 6.0; Media Cybernetics, Inc.).

Luciferase report assay. JASPAR database (jaspar.genereg.net/) was used to predict the binding sites of HOXB5 and ANGPT2. Luciferase report assay was performed to investigate the interaction between HOXB5 and ANGPT2 using Luciferase Reporter System (Promega Corporation). The cloning primers designed via Primer3Plus were as follows: ANGPT2 forward, 5'-GCATTTGCTGGAGGTCACAC-3' and reverse, 5'-AGCTGGAAGACATGCTCTGG-3'. The 3'-untranslated region (UTR) of ANGPT2 containing the seed sequence of wild-type (WT) or mutated (MUT) binding site of HOXB5 was cloned into pGL3 vectors (Promega Corporation) to generate pGL3-ANGPT2-3'UTR-WT and MUT luciferase reporter plasmids. Subsequently, the transfection of EC-9706 cells (2×10^5 cells/well) was performed with pGL3-based reporter constructs, as previously described (21). OPTI-MEM (49 μ l; Gibco; Thermo Fisher Scientific, Inc.) was used to dilute 1 μ l Lipofectamine 2000[®] reagent (Invitrogen; Thermo Fisher Scientific, Ltd.). Following 36 h transfection at 37°C, Dual-Luciferase Reporter assay system (Promega Corporation) was utilized to detect luciferase activity which was measured in comparison with *Renilla* luciferase activity using luciferase reporter assay substrate kit (Abcam).

Chromatin immunoprecipitation (CH-IP) assay. Formaldehyde (1%; Sigma-Aldrich; Merck KGaA) was used to crosslink EC-9706 cells for 10 min at 37°C in PBS; the reaction was terminated by adding glycine (Beijing Solarbio Science & Technology Co., Ltd.). A total of 300 μ l SDS lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl pH 8.0) was used to lyse 2×10^6 cells at room temperature for 10 min. The lysed cells were subjected to sonication (60 Hz) in ice water for 10 min. The resulting sonicated fragments were 200-1,000 bp in length. Following sonication, the samples were centrifuged at 13,000 \times g for 10 min at 4°C. Subsequently, the supernatant (100 μ g) were pre-absorbed by 50 μ l protein

G beads and was incubated with magnetic beads conjugated to 5 μ g anti-ANGPT2 (1 μ l/mg; cat. no. ab276042; Abcam), anti-HOXB5 (1/100; cat. no. ab229345; Abcam) or anti-rabbit IgG antibodies (1/100; cat. no. ab172730; Abcam) at 4°C for 2 h. The magnetic beads were then rinsed four times with lysis buffer, twice with LiCl buffer, and three times with Tris-EDTA buffer. The bound immunocomplex was eluted by adding 300 μ l of fresh elution buffer [10 mM Tris; 1 mM EDTA, (pH 8.0)]. Subsequently, 20 μ l 5 M NaCl was mixed with the eluted product, which was incubated at 65°C overnight to reverse the crosslinking and the purification of immunoprecipitated DNA was conducted using a CH-IP DNA purification kit (cat. no. D0033; Beyotime Biotechnology Institute) and the enrichment of ANGPT2 was detected using RT-qPCR. Primer sequences were as follows: ANGPT2 forward, 5'-TGTTCCAGAACCTTGGTGGGAAT-3' and reverse, 5'-AGTTCTGAGTATTGTGGCAGC-3' and GAPDH forward, 5'-AATGGGCAGCCGTTAGGAAA-3' and reverse 5'-GCGCCCAATACGACCAAATC-3'.

Bioinformatics analysis. Gene Expression Profiling Interactive (GEPIA) Analysis 2 database (gepia2.cancer-pku.cn/#index) was used to explore the expression of ANGPT2 and HOXB5 in esophageal cancer and the association between the expression levels of candidate genes and survival rate for patients with esophageal cancer. The key words ANGPT2 and HOXB5 were utilized as input. 'Expression DIY' and 'Survival analysis' in the Expression Analysis function were chosen for the analysis of The Cancer Genome Atlas and Genotype-Tissue expression data.

Statistical analysis. All experiments were repeated three times. All data collected from experiments are presented as mean \pm standard deviation and were analyzed with SPSS 11.0 software (SPSS, Inc.). Unpaired Student's t-test was used to analyze differences between 2 groups and one-way analysis of variance followed by Tukey's post hoc test was adopted to analyze differences among ≥ 3 groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

ANGPT2 is upregulated in esophageal cancer tissue and cell lines and is associated with poor patient prognosis. According to GEPIA database, ANGPT2 was upregulated in patients with esophageal cancer (Fig. 1A). Data from GEPIA database also demonstrated that ANGPT2 upregulation was significantly associated with low overall survival rate of patients with esophageal cancer (Fig. 1B). Compared with HEEC, mRNA and protein expression levels of ANGPT2 were enhanced in KYSE-70, KYSE-30 and EC-9706 cells (Fig. 1C and D). EC-9706 cells had the highest expression of ANGPT2 and were therefore selected for subsequent experiments. The aforementioned results suggested that ANGPT2 was upregulated in esophageal cancer cells and this led to lower overall survival rate.

ANGPT2 silencing inhibits proliferation of esophageal cancer. To knock down ANGPT2, shRNA targeting ANGPT2 was used to transfect EC-9706. RT-qPCR and

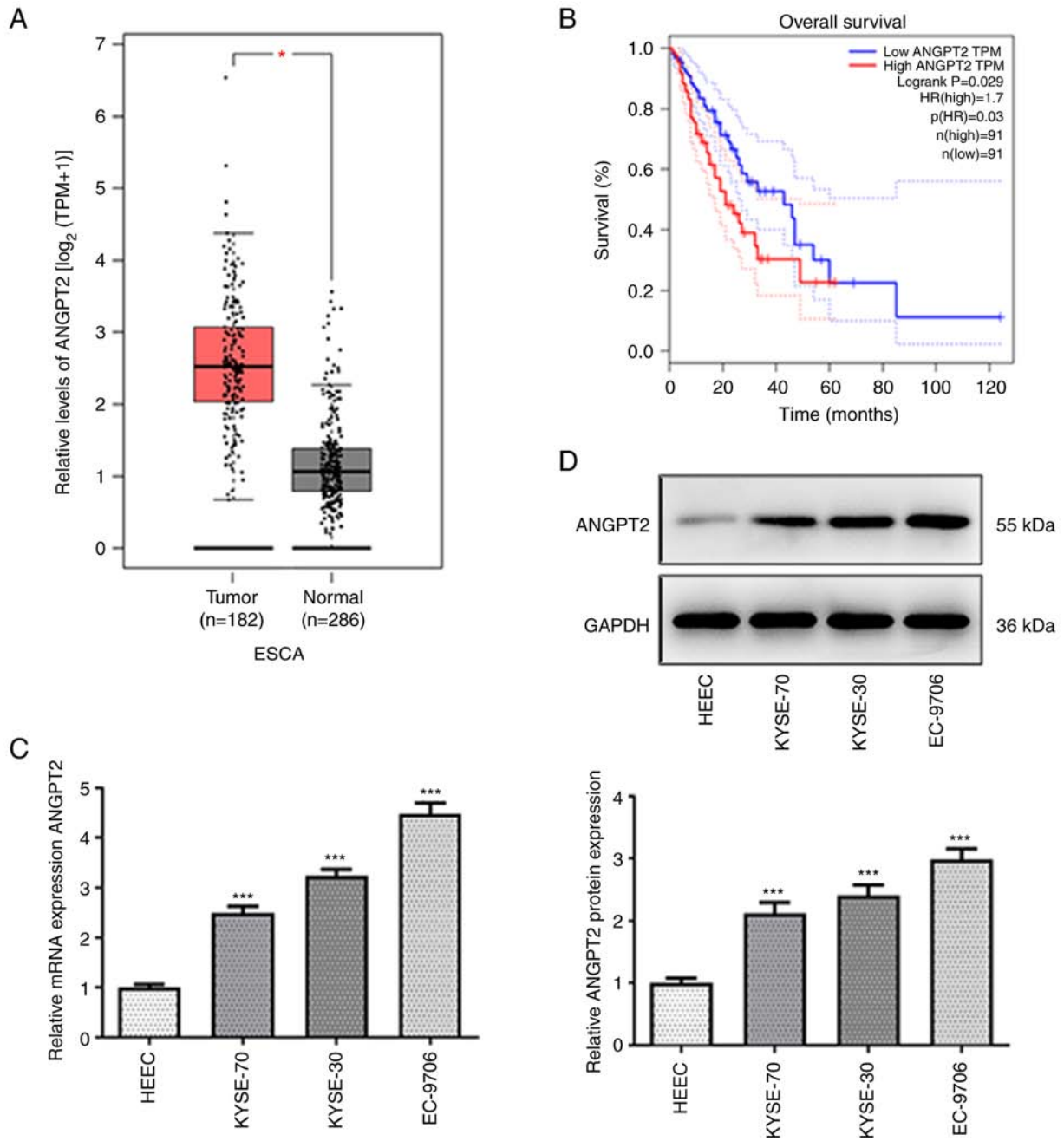


Figure 1. ANGPT2 upregulation in esophageal cancer tissue and cell lines and is associated with poor patient prognosis. (A) ANGPT2 was upregulated in patients with esophageal cancer. $^*P<0.05$ vs. normal. (B) ANGPT2 upregulation was significantly associated with low overall survival rate of patients with esophageal cancer. (C) Relative mRNA expression of ANGPT2 in normal HEEC and esophageal cancer cell lines was detected using reverse transcription-quantitative PCR. (D) Protein expression of ANGPT2 in normal HEEC and esophageal cancer cell lines was detected using western blotting. $^{***}P<0.001$ vs. HEEC. ANGPT2, angiopoietin-2; ESCA, esophageal carcinoma; HEEC, human esophageal epithelial cell; TPM, transcripts per million.

western blot analysis indicated that the mRNA and protein expression levels of ANGPT2 in EC-9706 cells were significantly decreased following transfection with sh-ANGPT2 plasmids (Fig. 2A and B). EC-9706 cells transfected with shRNA-ANGPT2#2 showed low ANGPT2 expression compared with shRNA-ANGPT2#1. Therefore, subsequent experiments were performed on EC-9706 cells transfected with shRNA-ANGPT2#2.

Viability, proliferation and colony formation of esophageal cancer cells were evaluated. The viability of EC-9706 cells was significantly decreased at 48 and 72 h following transfection with shRNA-ANGPT2 (Fig. 2C). Likewise, ANGPT2

silencing had suppressive effects on proliferation and colony formation of EC-9706 cells (Fig. 2D and E).

ANGPT2 silencing inhibits metastasis and angiogenesis of esophageal cancer cells. Wound healing and Transwell assays were performed to investigate the migration and invasion of ANGPT2-silenced EC-9706 cells. The relative migration rate and number of invaded EC-9706 cells were significantly decreased following transfection with shRNA-ANGPT2, revealing that ANGPT2 silencing inhibited metastasis of esophageal cancer cells (Fig. 3A-D). In addition, the expression levels of epithelial-mesenchymal transition

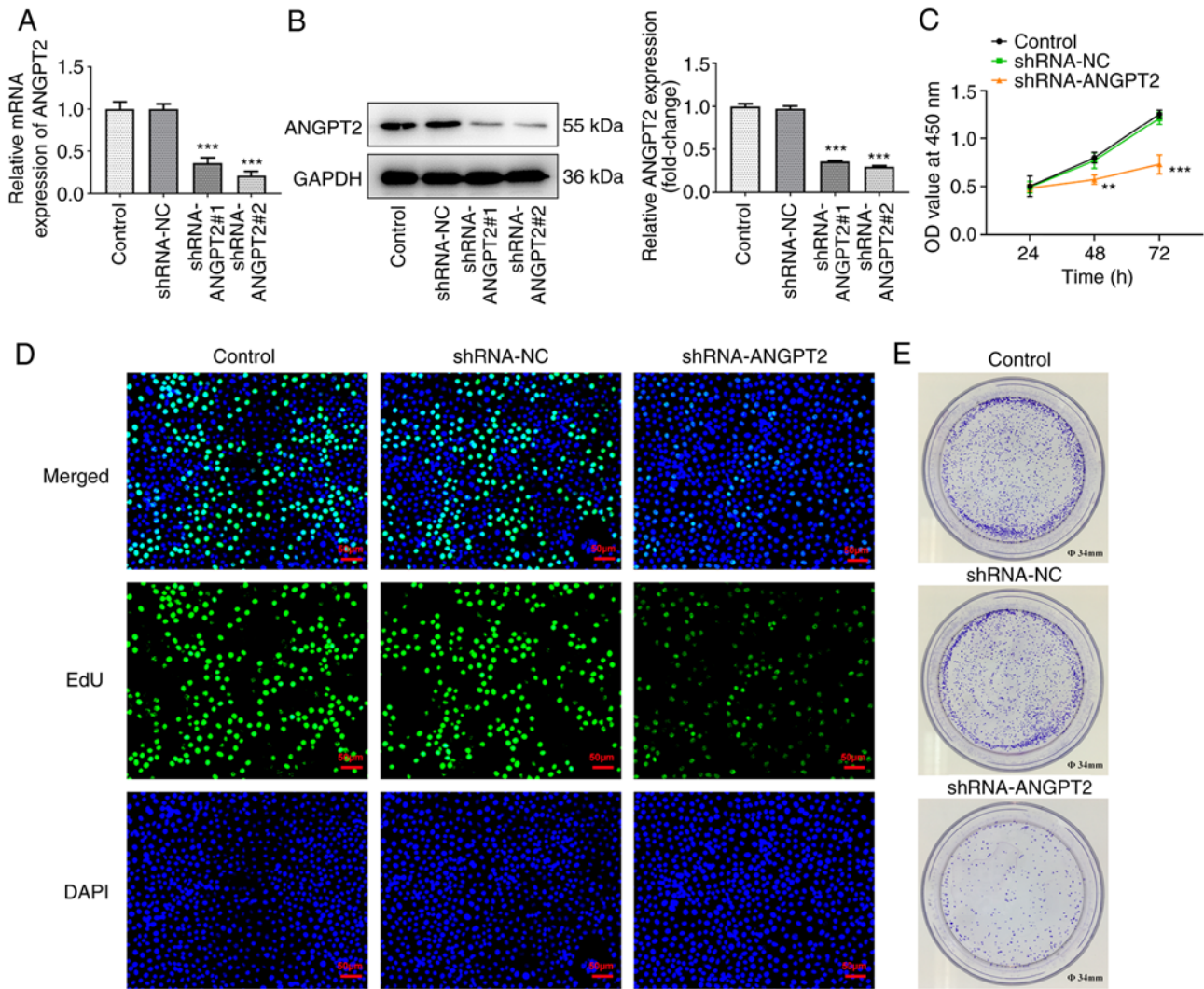


Figure 2. ANGPT2 silencing inhibits proliferation of EC-9706 esophageal cancer cells. (A) Relative mRNA expression of ANGPT2 was detected using reverse transcription-quantitative PCR. (B) Protein expression of ANGPT2 was detected using western blot analysis. *** $P < 0.001$ vs. shRNA-NC. (C) Viability of EC-9706 cells was detected using Cell Counting Kit-8. ** $P < 0.01$ and *** $P < 0.001$ vs. control. (D) Proliferation of EC-9706 cells was detected using EDU staining assay (scale bar, 50 μm). (E) Colony formation assay of EC-9706 cells (scale bar, 34 mm). ANGPT2, angiopoietin-2; shRNA, short harpin RNA; NC, negative control; OD, optical density.

(EMT)-associated proteins and biomarkers (Vimentin) were measured by western blot assay. ANGPT2 silencing upregulated E-cadherin but downregulated N-cadherin and Vimentin expression (Fig. 3E). Moreover, tube formation analysis indicated that the number of tubes was decreased following transfection with shRNA-ANGPT2, indicating that angiogenesis of esophageal cancer cells was inhibited by ANGPT2 silencing (Fig. 3F).

HOXB5 transcription activates ANGPT2. JASPAR database was used to predict the binding sites of transcription factor HOXB5 and ANGPT2 promoters (Fig. 4A). According to GEPIA database, HOXB5 had a high expression in tissue of patients with esophageal cancer compared with normal tissue, while its upregulation had no significant association with low overall survival rate of patients with esophageal cancer (Fig. 4B and C). In addition, the mRNA and protein levels of HOXB5 in EC-9706 cells were increased compared with those in HEECs (Fig. 4D and E).

To increase expression of HOXB5, EC-9706 cells were transfected with pcDNA3.1-HOXB5 plasmids. Both mRNA and protein levels of HOXB5 were enhanced in HOXB5-overexpressing EC-9706 cells compared with pcDNA3.1 group (Fig. 4F and G). Moreover, ANGPT2 promoters were activated by the transcription factor HOXB5, as suggested by the strong luciferase activity observed in the ANGPT2-WT + pcDNA3.1-HOXB5 group (Fig. 4H). To validate the binding ability of HOXB5 and ANGPT2 promoters, CH-IP assay was performed with HOXB5 antibody. ANGPT2 was enriched in anti-HOXB5, indicating that HOXB5 bound to ANGPT2 promoters (Fig. 4I).

Overexpression of transcription factor HOXB5 reverses effects of ANGPT2 silencing on esophageal cancer cells. The mRNA and protein expression levels of ANGPT2, which were decreased in the shRNA-ANGPT2 group, were partly recovered in the shRNA-ANGPT2 + pcDNA3.1-HOXB5 group (Fig. 5A). The viability, proliferation and colony formation, which were

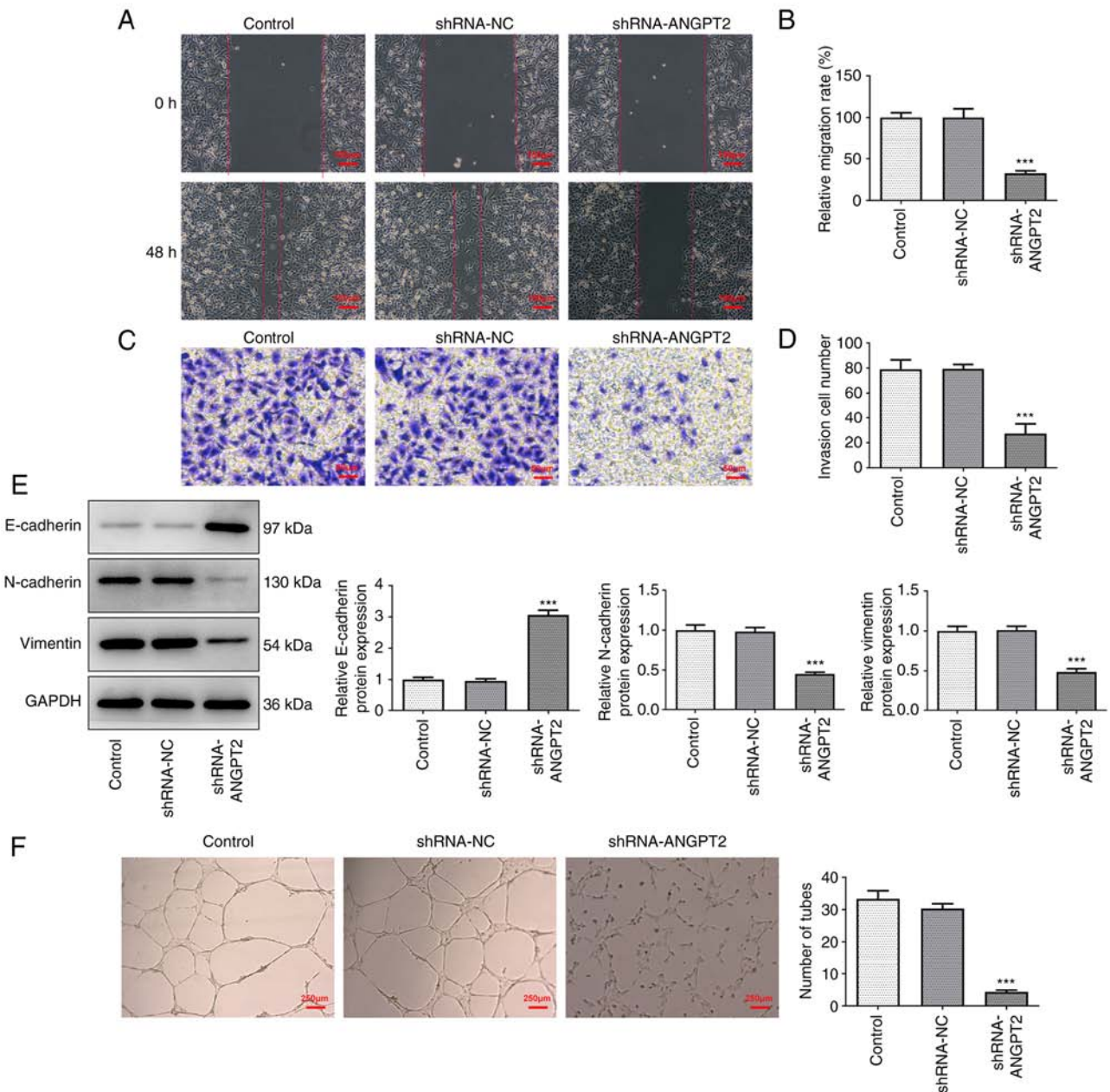


Figure 3. ANGPT2 silencing inhibits metastasis and angiogenesis of EC-9706 esophageal cancer cells. (A) Wound healing assay was used to determine (B) migration of EC-9706 cells (scale bar, 100 μ m). (C) Transwell assay was used to determine (D) invasion of EC-9706 cells (scale bar, 50 μ m). (E) Expression levels of epithelial-mesenchymal transition-associated proteins were detected using western blot analysis. (F) Angiogenic effect was investigated using tube formation analysis (scale bar, 250 μ m). *** P <0.001 vs. shRNA-NC. ANGPT2, angiopoietin-2; shRNA, short harpin RNA; NC, negative control.

decreased in the shRNA-ANGPT2 group, were partially restored in the shRNA-ANGPT2 + pcDNA3.1-HOXB5 group, revealing that HOXB5 overexpression could reverse the effect of ANGPT2 silencing (Fig. 5B-D).

The migration and invasion of EC-9706 cells were diminished following transfection with shRNA-ANGPT2; this effect was reversed by HOXB5 overexpression (Fig. 6A-D). Moreover, ANGPT2 silencing upregulated E-cadherin expression and downregulated the expression levels of N-cadherin and Vimentin, whereas HOXB5 overexpression partially abolished the aforementioned effects of ANGPT2 silencing (Fig. 6E). Furthermore, the decreased number of tubes in ANGPT2-silenced EC-9706 cells was increased following HOXB5 overexpression, suggesting that HOXB5

overexpression enhanced angiogenesis of esophageal cancer cells (Fig. 6F).

Overexpression of transcription factor HOXB5 abolishes the inactivation of ERK/AKT signaling pathway induced by ANGPT2 silencing. To understand the effects of ANGPT2 silencing on ERK/AKT signaling pathway, the expression levels of ERK/AKT signaling pathway-associated proteins, such as p-ERK, p-AKT, ERK and AKT, were measured using western blotting. The decreased expression levels of p-ERK and p-AKT in ANGPT2-silenced EC-9706 cells were upregulated after overexpressing HOXB5. However, expression levels of ERK and AKT remained unchanged following transfection with shRNA-ANGPT2 and pcDNA3.1-HOXB5 (Fig. 7). The

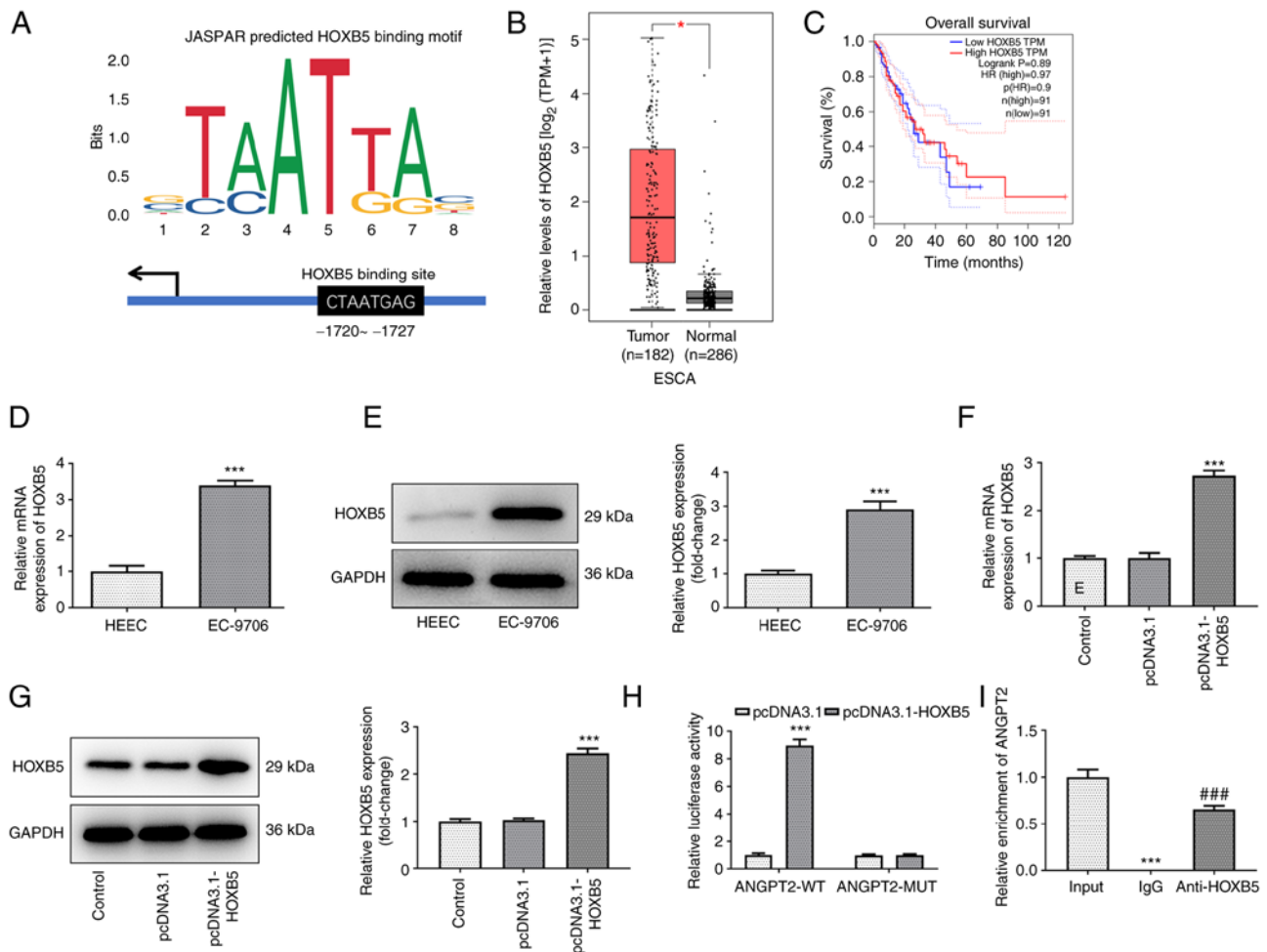


Figure 4. HOXB5 transcription activates ANGPT2. (A) JASPAR-predicted HOXB5 binding motif. (B) HOXB5 was upregulated in patients with esophageal cancer. * $P < 0.05$. (C) HOXB5 upregulation was not significantly associated with low overall survival rate of patients with esophageal cancer. (D) Relative mRNA expression of HOXB5 was detected using RT-qPCR. (E) Protein expression of HOXB5 was detected using western blot analysis. *** $P < 0.001$ vs. HEEC. Relative (F) mRNA and (G) protein expression levels of HOXB5 in HOXB5-overexpressing EC-9706 cells were detected using RT-qPCR and western blot analysis. *** $P < 0.001$ vs. pcDNA3.1. (H) Relative luciferase activity was detected using luciferase reporter assay. *** $P < 0.001$ vs. ANGPT2-WT + pcDNA3.1. (I) Relative enrichment of ANGPT2 was detected using chromatin immunoprecipitation. *** $P < 0.001$ vs. input; ### $P < 0.001$ vs. IgG. HOXB5, homeobox B5; ANGPT2, angiopoietin-2; RT-qPCR, reverse transcription-quantitative PCR; ESCA, esophageal carcinoma; WT, wild type; MUT, mutated; TPM, transcripts per million.

above-mentioned results indicated that HOXB5 overexpression blocked the inhibitory effect of ANGPT2 silencing on ERK/AKT signaling pathway.

Discussion

To the best of our knowledge, the present study is the first to investigate the role of HOXB5 and ANGPT2 in the malignant progression of esophageal cancer. Firstly, the expression levels of HOXB5 and ANGPT2 in esophageal cancer cells were detected. Subsequently, functional experiments were conducted to explore the effects of ANGPT2 silencing on the proliferation and colony formation of esophageal cancer cells. In the present study, ANGPT2 and HOXB5 were upregulated in esophageal cancer cells; ANGPT2 upregulation was significantly associated with the low overall survival rate of patients with esophageal cancer. Moreover, ANGPT2 silencing inhibited the viability, proliferation, colony formation, migration, invasion and angiogenesis of esophageal cancer cells. In addition, the HOXB5 transcription factor was demonstrated to activate ANGPT2, whereas HOXB5

overexpression reversed the effect of ANGPT2 silencing on the proliferation, metastasis and angiogenesis of esophageal cancer cells. Furthermore, the inhibition of the ERK/AKT signaling pathway caused by ANGPT2 silencing was also reversed by HOXB5 overexpression.

In recent years, a number of studies have been performed to explore the role of ANGPT2 in cancer (22-24). For example, miR-145-5p overexpression exerts inhibitory effects on the proliferation, migration and invasion of gastric cancer cells via the ANGPT2 axis (22). In addition, the insulin gene enhancer protein ISL2 induces angiogenesis to promote malignant transformation via regulating ANGPT2 (23). Moreover, ANGPT2 may serve as a potential therapeutic target for anti-angiogenic therapy (24). In the present study, ANGPT2 was upregulated in esophageal cancer cells and this was associated with low overall survival of patients with esophageal cancer. Additionally, the viability, proliferation, colony formation, migration, invasion and angiogenesis were inhibited in ANGPT2-silenced EC-9706 cells.

Several studies have suggested that HOXB5 may serve a key role in the regulation of tumor progression (25,26). For

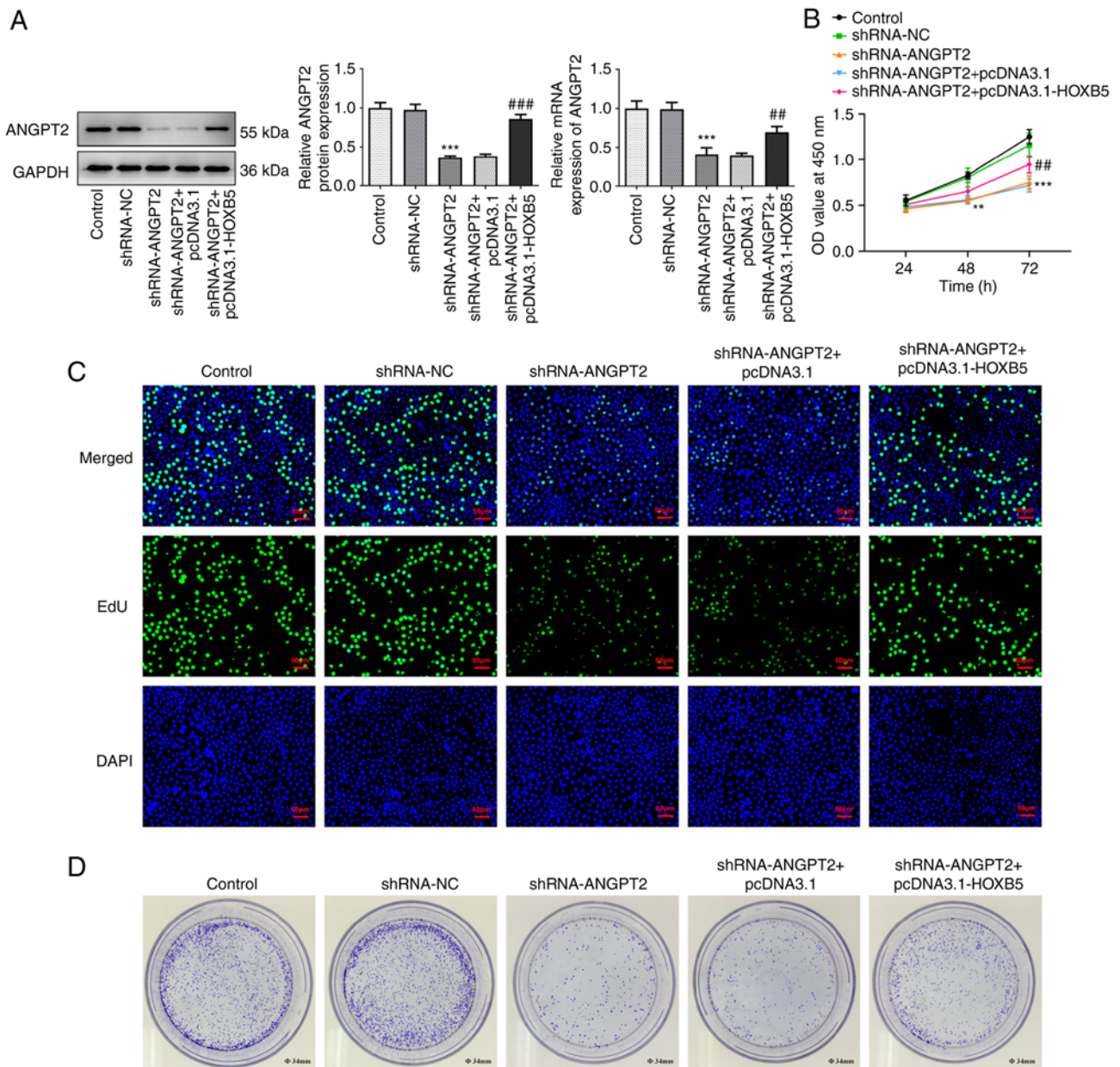


Figure 5. Overexpression of transcription factor HOXB5 reverses the inhibitory effects of ANGPT2 silencing on proliferation of EC-9706 esophageal cancer cells. (A) Relative mRNA and protein expression levels of ANGPT2 were detected using reverse transcription-quantitative PCR and western blot analysis. (B) Viability of EC-9706 cells was detected using Cell Counting Kit-8. **P<0.01 and ***P<0.001 vs. shRNA-NC. ***P<0.01 and ***P<0.001 vs. shRNA-ANGPT2+pcDNA3.1. (C) Proliferation was detected using EdU staining (scale bar, 50 μm). (D) Colony forming ability was detected using colony formation assay (scale bar, 34 mm). HOXB5, homeobox B5; ANGPT2, angiotensin-2; shRNA, short harpin RNA; OD, optical density; NC, negative control.

example, HOXB5 exerts promotive effects on the proliferation, migration and invasion of pancreatic cancer cells (27). Lee *et al* (15) suggested that HOXB5 serves as an oncogenic driver in head and neck squamous cell carcinoma. In the present study, HOXB5 was upregulated in esophageal cancer cells. Data from JASPAR database predicted the binding between transcription factor HOXB5 and ANGPT2, which was verified by luciferase reporter and CH-IP assay. Moreover, the effect of ANGPT2 silence on the proliferation, metastasis and angiogenesis of esophageal cancer cells were reversed following HOXB5 overexpression.

A previous study indicated that stimulation of ERK/AKT pathway signaling enhances proliferation, survival and metabolism of cancer cells (28). Zhou *et al* (29) demonstrated that

blockade of the ERK/AKT pathway inhibits human endometriosis progression. Moreover, activation of ERK/AKT pathway promotes proliferation and migration of renal cancer cells (30). In the present study, ERK/AKT signaling was inhibited by ANGPT2 silencing, while HOXB5 overexpression partially abolished the effects of ANGPT2 silencing.

There are some limitations in the present study. The present study was performed only on the EC-9706 cell line; other types of esophageal cancer cell should be investigated in future as the role of HOXB5 may be different in the different types of esophageal cancer. Moreover, the effect of downregulation of HOXB5 on ANGPT2 in esophageal cancer need to be explored in future investigations. Furthermore, the EC-9706 cell line displayed the highest ANGPT2 expression levels and

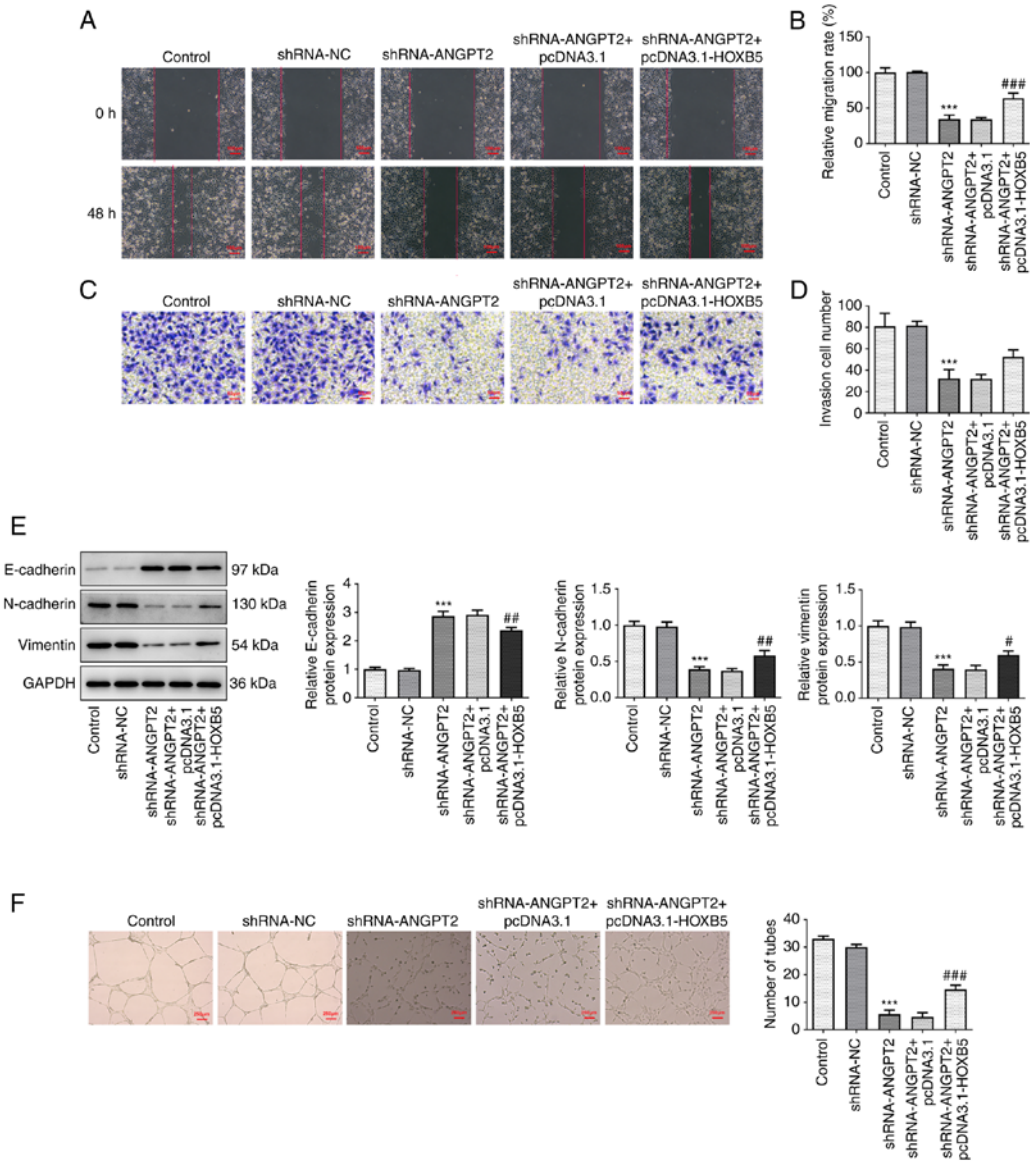


Figure 6. Overexpression of transcription factor HOXB5 reverses the inhibitory effects of ANGPT2 silencing on the metastasis and angiogenesis of EC-9706 esophageal cancer cells. (A) Wound healing assay was used to detect (B) cell migration (scale bar, 100 μ m). (C) Transwell assay was used to determine (D) cell invasion (scale bar, 50 μ m). (E) Expression levels of epithelial-mesenchymal transition-associated proteins were detected using western blot analysis. (F) Angiogenic effect measured using tube formation analysis (scale bar, 250 μ m). *** P <0.001 vs. shRNA-NC, ** P <0.05, ## P <0.01 and ### P <0.001 vs. shRNA-ANGPT2 + pcDNA3.1. HOXB5, homeobox B5; ANGPT2, angiotensinogen-converting enzyme 2; shRNA, short hairpin RNA; NC, negative control.

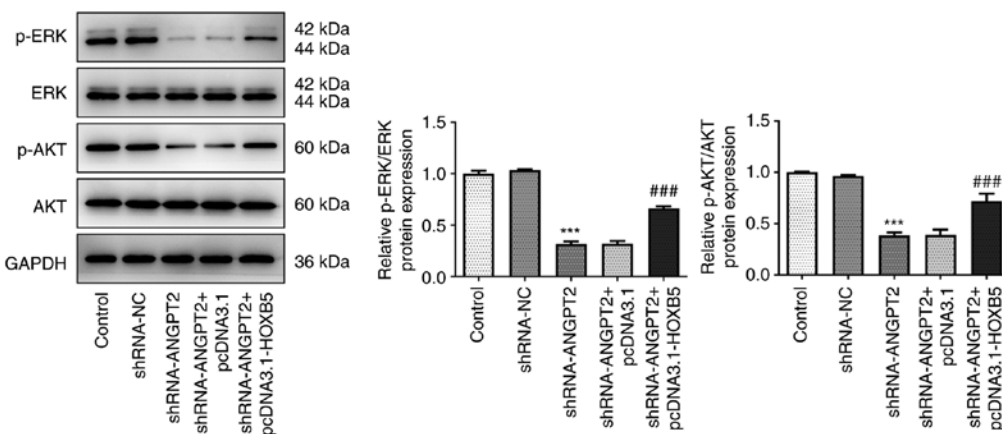


Figure 7. Overexpression of transcription factor HOXB5 reverses the inhibitory effects of ANGPT2 silencing on the ERK/AKT signaling pathway. Expression levels of signaling pathway-associated proteins were detected using western blot analysis. *** P <0.001 vs. shRNA-NC. ### P <0.001 vs. shRNA-ANGPT2 + pcDNA3.1. HOXB5, homeobox B5; ANGPT2, angiotensinogen-converting enzyme 2; sh, short hairpin; NC, negative control; p-, phosphorylated.

this should also be investigated in future work. To the best of our knowledge, HOXB5/ANGPT2 have not been investigated for use in the treatment of other types of cancer.

In conclusion, ANGPT2 silencing inhibited the proliferation, migration, invasion and angiogenesis of esophageal cancer cells via targeting HOXB5 and blocking the ERK/AKT signaling pathway, suggesting that ANGPT2/HOXB5 may be potential therapeutic targets for the treatment of angiogenesis abnormality and metastasis of esophageal cancer.

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Availability of data and materials

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

Authors' contributions

SG designed the experiments and wrote the paper. JL performed the experiments, participated in study design and wrote the manuscript. All authors have read and approved the final manuscript. JL and SG confirm the authenticity of all the raw data.

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.

Reference

- Pakzad R, Mohammadian-Hafshejani A, Khosravi B, Soltani S, Pakzad I, Mohammadian M, Salehiniya H and Momenimovahed Z: The incidence and mortality of esophageal cancer and their relationship to development in Asia. *Ann Transl Med* 4: 29, 2016.
- Tan HZ, Lin WJ, Huang JQ, Dai M, Fu JH, Huang QH, Chen WM, Xu YL, Ye TT, Lin ZY, *et al*: Updated incidence rates and risk factors of esophageal cancer in Nan'ao Island, a coastal high-risk area in southern China. *Dis Esophagus* 30: 1-7, 2017.
- Lei S, Yang L and Li Y: Epidemiological characteristics and changing trends of esophageal cancer diagnosed by gastroscopy in Xijing Hospital from 2016 to 2020. *J Hebei Med Univ* 43: 150-154, 2022 (In Chinese).
- Pennathur A, Gibson MK, Jobe BA and Luketich JD: Oesophageal carcinoma. *Lancet* 381: 400-412, 2013.
- Dvoretzkii Slu, Levchenko EV, Karachun AM, Komarov IV, Pelipas' Iu V, Avanesian AA, Khandogin NV and Tiuriaeva EI: Experience of the use of endovideotechnology in surgical treatment of esophageal cancer. *Vestn Khir Im I I Grek* 173: 54-59, 2014 (In Russian).
- Sugimura K, Miyata H, Tanaka K, Takahashi T, Kurokawa Y, Yamasaki M, Nakajima K, Takiguchi S, Mori M and Doki Y: High infiltration of tumor-associated macrophages is associated with a poor response to chemotherapy and poor prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer. *J Surg Oncol* 111: 752-759, 2015.
- Zhu Z, Wang H, Pang Y, Hu H, Zhang H and Wang W: Exosomal long non-coding RNA UCA1 functions as growth inhibitor in esophageal cancer. *Aging (Albany NY)* 12: 20523-20539, 2020.
- Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, *et al*: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997.
- Saharinen P, Eklund L and Alitalo K: Therapeutic targeting of the angiopoietin-TIE pathway. *Nat Rev Drug Discov* 16: 635-661, 2017.
- Ladeira K, Macedo F, Longatto-Filho A and Martins SF: Angiogenic factors: Role in esophageal cancer, a brief review. *Esophagus* 15: 53-58, 2018.
- Urosevic J, Blasco MT, Llorente A, Bellmunt A, Berenguer-Llargo A, Guiu M, Canellas A, Fernandez E, Burkov I, Clapes M, *et al*: ERK1/2 signaling induces upregulation of ANGPT2 and CXCR4 to mediate liver metastasis in colon cancer. *Cancer Res* 80: 4668-4680, 2020.
- Lauret Marie Joseph E, Laheurte C, Jary M, Boullerot L, Asgarov K, Gravelin E, Bouard A, Rangan L, Dosset M, Borg C and Adotévi O: Immunoregulation and clinical implications of ANGPT2/TIE2(+) M-MDSC signature in non-small cell lung cancer. *Cancer Immunol Res* 8: 268-279, 2020.
- Zhang B, Li N and Zhang H: Knockdown of homeobox B5 (HOXB5) inhibits cell proliferation, migration, and invasion in non-small cell lung cancer cells through inactivation of the Wnt/ β -catenin pathway. *Oncol Res* 26: 37-44, 2018.
- Hong CS, Jeong O, Piao Z, Guo C, Jung MR, Choi C and Park YK: HOXB5 induces invasion and migration through direct transcriptional up-regulation of β -catenin in human gastric carcinoma. *Biochem J* 472: 393-403, 2015.
- Lee K, Chang JW, Oh C, Liu L, Jung SN, Won HR, Kim YI, Rha KS and Koo BS: HOXB5 acts as an oncogenic driver in head and neck squamous cell carcinoma via EGFR/Akt/Wnt/ β -catenin signaling axis. *Eur J Surg Oncol* 46: 1066-1073, 2020.
- Li ZX, Wu G, Jiang WJ, Li J, Wang YY, Ju XM and Yin YT: HOXB5 promotes malignant progression in pancreatic cancer via the miR-6732 pathway. *Cell Cycle* 19: 233-245, 2020.
- Feng W, Huang W, Chen J, Qiao C, Liu D, Ji X, Xie M, Zhang T, Wang Y, Sun M, *et al*: CXCL12-mediated HOXB5 overexpression facilitates colorectal cancer metastasis through transactivating CXCR4 and ITGB3. *Theranostics* 11: 2612-2633, 2021.
- Zhang J, Zhang S, Li X, Zhang F and Zhao L: HOXB5 promotes the progression of breast cancer through wnt/ β -catenin pathway. *Pathol Res Pract* 224: 153117, 2021.
- Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25: 402-408, 2001.
- Liang J, Li H, Han J, Jiang J, Wang J, Li Y, Feng Z, Zhao R, Sun Z, Lv B and Tian H: Mex3a interacts with LAMA2 to promote lung adenocarcinoma metastasis via PI3K/AKT pathway. *Cell Death Dis* 11: 614, 2020.
- Chang J, Hu X, Nan J, Zhang X and Jin X: HOXD9-induced SCNN1A upregulation promotes pancreatic cancer cell proliferation, migration and predicts prognosis by regulating epithelial-mesenchymal transformation. *Mol Med Rep* 24: 819, 2021.
- Zhou K, Song B, Wei M, Fang J and Xu Y: MiR-145-5p suppresses the proliferation, migration and invasion of gastric cancer epithelial cells via the ANGPT2/NOD-LIKE-RECEPTOR axis. *Cancer Cell Int* 20: 416, 2020.
- Qi L, Wang ZY, Shao XR, Li M, Chen SN, Liu XQ, Yan S, Zhang B, Zhang XD, Li X, *et al*: ISL2 modulates angiogenesis through transcriptional regulation of ANGPT2 to promote cell proliferation and malignant transformation in oligodendroglioma. *Oncogene* 39: 5964-5978, 2020.
- Xie JY, Wei JX, Lv LH, Han QF, Yang WB, Li GL, Wang PX, Wu SB, Duan JX, Zhuo WF, *et al*: Angiopoietin-2 induces angiogenesis via exosomes in human hepatocellular carcinoma. *Cell Commun Signal* 18: 46, 2020.
- Lee JY, Hur H, Yun HJ, Kim Y, Yang S, Kim SI and Kim MH: HOXB5 promotes the proliferation and invasion of breast cancer cells. *Int J Biol Sci* 11: 701-711, 2015.
- Tucci R, Campos MS, Matizonkas-Antonio LF, Durazzo M, Pinto Junior Ddos S and Nunes FD: HOXB5 expression in oral squamous cell carcinoma. *J Appl Oral Sci* 19: 125-129, 2011.

27. Gao Y, Fei X, Kong L and Tan X: HOXB5 promotes proliferation, migration, and invasion of pancreatic cancer cell through the activation of the GSK3 β / β -catenin pathway. *Anticancer Drugs* 31: 828-835, 2020.
28. Asati V, Mahapatra DK and Bharti SK: PI3K/Akt/mTOR and Ras/Raf/MEK/ERK signaling pathways inhibitors as anticancer agents: Structural and pharmacological perspectives. *Eur J Med Chem* 109: 314-341, 2016.
29. Zhou CF, Liu MJ, Wang W, Wu S, Huang YX, Chen GB, Liu LM, Peng DX, Wang XF, Cai XZ, *et al*: miR-205-5p inhibits human endometriosis progression by targeting ANGPT2 in endometrial stromal cells. *Stem Cell Res Ther* 10: 287, 2019.
30. Zhu R, Ge J, Ma J and Zheng J: Carcinoembryonic antigen related cell adhesion molecule 6 promotes the proliferation and migration of renal cancer cells through the ERK/AKT signaling pathway. *Transl Androl Urol* 8: 457-466, 2019.



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