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
Transfection, total RNA isolated from cells using TRIzol®. Cells were placed in a 6-well plate (6x10⁴ cells/well). Following reverse transcription-quantitative PCR (RT-qPCR). EC-9706 are not be provided as the company did not provide them. Experiments. Sequence fragments that interfere with ANGPT2. Following 48 h incubation, cells were collected for subsequent experiments. Reverse transcription-quantitative PCR (RT-qPCR). EC-9706 cells at 90% confluence were inoculated into 6-well plates in logarithmic growth phase were inoculated into 6-well plates (6x10⁴ cells/well). EC-9706 cells were plated in a 6-well plate (6x10⁴ cells/well). Following transfection, total RNA isolated from cells using TRIZol® reagent (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 µl 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 µg), as well as corresponding negative control (shRNA-NC; 50 nM) and pcDNA3.1-NC (2 µg) 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. Subsequently, 10 µl CCK-8 reagent (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 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).

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 µg/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 µl 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 µM 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-µl pipette tip was applied. After washing three times with PBS, the cells were then incubated in serum-free DMEM for 4 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 (1x10^5 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 µl) were seeded into a precooled 96-well plate (1.5x10^5 cells/well) before addition of 100 µl/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'-GCA TTT GCT GGA GGT CAC AC-3' and reverse, 5'-AGC TGG AAG ACA TGC TCT GG-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-WT and MUT luciferase reporter plasmids. Subsequently, the transfection of EC-9706 cells (2x10^5 cells/well) was performed with pGL3-based reporter constructs, as previously described (21).

**Statistical analysis.** All experiments were repeated three times. All data collected from experiments are presented as mean ± 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
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
(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...
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).
aforementioned 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 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
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

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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, angiopoietin-2; shRNA, short hairpin RNA; OD, optical density; NC, negative control.
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 µm). (C) Transwell assay was used to determine (D) cell invasion (scale bar, 50 µm). (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 µm). ***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, angiopoietin-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, angiopoietin-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.

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