INTRODUCTION

Esophageal cancer is one of the most common gastrointestinal tumors worldwide, ranking seventh in incidence and sixth in cancer-related mortality in 2018. The main types of esophageal cancers are ESCC and esophageal adenocarcinoma (EAC). ESCC is the predominant histological type of esophageal cancer in China. The geographical variation in the incidence is striking, exhibiting a high prevalence in the Taihang mountain region in north-central China, such as Cixian in Hebei, Henan, and Shanxi provinces. Although there have been great advances in the treatment of esophageal cancer, patients with ESCC experience malignant proliferation and invasion, leading to poor survival (5-y survival rate <30%). Therefore, understanding the ESCC progression mechanism to improve the clinical outcome of patients with ESCC is essential.

SNHG17, as an EMT-related IncRNA, promotes the expression of c-Myc by binding to c-Jun in esophageal squamous cell carcinoma

Dysregulation of long noncoding RNA SNHG17 is associated with the occurrence of several tumors; however, its role in esophageal squamous cell carcinoma (ESCC) remains obscure. The present study demonstrated that SNHG17 was upregulated in ESCC tissues and cell lines, induced by TGF-β1, and associated with poor survival. It is also involved in the epithelial-to-mesenchymal transition (EMT) process. The mechanism underlying SNHG17-regulated c-Myc was detected by RNA immunoprecipitation, RNA pull-down, chromatin immunoprecipitation, and luciferase reporter assays. SNHG17 was found to directly regulate c-Myc transcription by binding to c-Jun protein and recruiting the complex to specific sequences of the c-Myc promoter region, thereby increasing its expression. Moreover, SNHG17 hyperactivation induced by TGF-β1 results in PI3K/AKT pathway activation, promoting cells EMT, forming a positive feedback loop. Furthermore, SNHG17 facilitated ESCC tumor growth in vivo. Overall, this study demonstrated that the SNHG17/c-Jun/c-Myc axis aggravates ESCC progression and EMT induction by TGF-β1 and may serve as a new therapeutic target for ESCC.

KEYWORDS

c-Myc, EMT, esophageal squamous cell carcinoma, metastasis, SNHG17
Long noncoding RNAs (lncRNAs) are RNA molecules with length exceeding 200 nucleotides (nt) and account for >76% of noncoding RNAs (ncRNAs). The lncRNAs have been implicated in a wide repertoire of cellular processes, including cell differentiation, cell cycle regulation, cell apoptosis, and epithelial-to-mesenchymal transition (EMT). Recent studies have shown that lncRNAs serve as master regulators in tumor progression and play a key role in encouraging or combating carcinogenesis, including ESCC. The oncogenic role of a lncRNA, small nucleolar RNA host gene 17 (SNHG17), has been reported, in which it annotates cancer-associated lncRNA functionally and is mechanistically linked to disease pathogenesis in colon cancer, prostate cancer, gastric cancer, and non–small-cell lung cancer. SNHG17 acts as a prognostic factor, promoting tumor proliferation and metastasis and is related to poor prognosis in these cancers. Nonetheless, how SNHG17 participates in the progress of ESCC is yet unknown.

EMT is a cellular process that regulates cancer cell invasion, metastatic potential, and resistance to radiation and chemotherapy. TGF-β acts as a potent EMT-inducing cytokine in promoting cancer cell invasion and metastasis. Moreover, TGF-β-induced lncRNAs have been shown to play crucial roles in various carcinogenic processes. For instance, long noncoding RNA activated by transforming growth factor-β (lncRNA-ATB) may be induced by TGF-β treatment to facilitate the malignant progression of hepatocellular carcinoma and breast cancer cells. TGF-β-activated lncRNA UCA1 promotes the proliferation of bladder transitional carcinoma cells through the non-canonical PI3K-AKT pathway. In prostate cancer, knockdown of SNHG17 weakened the EMT progression of cancer cells. Together, these studies suggested that lncRNA plays a major role in TGF-β-induced tumor invasion and metastasis. However, few studies have addressed the mechanism of interaction between lncRNA and TGF-β classical and non-classical pathways in ESCC. Additionally, SNHG17 promotes tumor invasion and metastasis in oral squamous cell carcinoma, gastric cancer, and other gastrointestinal tumors. Therefore, we speculated that SNHG17 is related to TGF-β-ESC; and whether TGF-β-regulated SNHG17 is involved in EMT needs further exploration.

In the current study, we aimed to investigate the biological function of SNHG17 and its role in TGF-β1-induced EMT in ESCC. Therefore, we unveiled the molecular mechanism underlying the pathogenesis of ESCC. These findings indicated that SNHG17 acts as a potential therapeutic strategy for ESCC.

2.2 Human ESCC tissue specimens

In total, 128 matched ESCC and paired para-cancerous tissues (at least 5 cm distal to tumor lesion) were collected from the Fourth Affiliated Hospital of Hebei Medical University, Shijiazhuang, China from May 2012 to December 2015. Clinical and survival data of patients were obtained from the clinical data registry (Table S1). Ethical approval for this study was obtained from the Ethics Committee of the Fourth Affiliated Hospital, Hebei Medical University.

2.3 Cell culture

Human ESCC cell lines (TE1, Eca109, Kyse150, Kyse170, and Yes2) were purchased from the American Type Culture Collection and maintained in RPMI-1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). When cells reached 70%-80% confluency, cells were stimulated with 10 ng/mL recombinant TGF-β1 (R&D Systems Inc) for 7 d. Then, total RNA was extracted and reverse transcribed into cDNA to examine SNHG17 expression.

2.4 Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from ESCC tissues and cells using TRIzol reagent (Invitrogen). Then, 1 μg of the total RNA was reverse transcribed into cDNA as detailed by the manufacture (Roche). Quantitative real-time PCR assay was performed using the SYBR qPCR Master Mix (Life Technology). Relative expression levels of lncRNA/mRNA were normalized to that of the housekeeping gene β-actin and quantitated by the comparative cycle threshold (Ct) method ($2^{ΔΔCt}$). Primers sequences and reaction assays for this study are displayed in Table S2.

2.5 Plasmid construction and cell transfection

Gene-specific shRNAs targeting SNHG17, control shRNAs, small interfering RNAs (siRNAs) targeting c-Jun, and siNC were synthesized by GenePharma Company. Overexpression plasmids containing pcDNA3.1-SNHG17 and pcDNA3.1-c-Jun, were synthesized by Genscript Company. SNHG17-MUT (Δ101-400 bp truncated mutant) overexpression vector was purchased from GenePharma. To construct expression vectors for His-tagged c-Jun, cDNAs encoding c-Jun or truncated versions (Δ1-242AA, Δ243-331AA) of c-Jun were synthesized by Generay and subcloned into the NdeI and Xhol sites of the PET-28a-His vector. The c-Myc promoter region (~998 to +73bp) and a truncation (c-Myc-P1 containing binding sites E1 and E2, ~335 to +73 bp) were cloned into the pGL3-basic vector, and named c-Myc-wild type (WT) and c-Myc-P1, respectively. Point mutations of c-Jun binding to pGL3-c-Myc-WT were promoted using a Q5® Site-Directed Mutagenesis Kit (New England Biolabs), and named c-Myc-MUT.
Lipofectamine 2000 (Invitrogen) was used to transfec Eca109 or Kyse150 cells inoculated into six-well plates and transfected, respectively, with plasmids or siRNA for 24 h. All sequences are shown in Table S3.

2.6 | Cell proliferation

MTS and colony forming assays were performed to determine the ability for cellular proliferation. For MTS assay, Eca109, Kyse150, and TE1 cells transfected with pcDNA3.1-SNHG17/shSNHG17 were seeded into 96-well plates. After 0, 24, 48, 72, or 96 h of incubation, assays were performed by adding 20 μL of the CellTiter 96® AQueous One Solution Reagent (Promega) directly to the culture wells, followed by incubation at 37°C for 4 h. Absorbance was recorded at 490 nm on a microplate reader (Thermo Fisher Scientific).

For the colony forming assay, Eca109, Kyse150 and TE1 cells were seeded into six-well plates at a density of 3000-5000 cells/well and cultured for 7 d. The surviving colonies were fixed in 4% paraformaldehyde and stained with 0.4% crystal violet solution and counted.

2.7 | Flow cytometry cell apoptosis assays

Eca109 and Kyse150 cells transfected with shSNHG17 were collected after 48 h and washed with PBS. Then, the cells were resuspended in 1x binding buffer at a concentration of 1 × 10⁶ cells/mL. After double staining with 5 μL fluorescein isothiocyanate (PE)-annexin V and 5 μL 7-amino-actinomycin (7-AAD; BD Pharmingen™), the cells were analyzed on a flow cytometer.

2.8 | Migration and invasion assays

For the cell migration assay, 1 × 10² infected Eca109, Kyse150 or TE1 cells were plated on non-Matrigel-coated in the upper chamber (Corning Company) with 200 μL of serum-free medium, and 600 μL of RPMI-1640 medium containing 20% serum was placed in the lower chamber. For the invasion assay, cells were seeded onto 50 μL 1x Matrigel® Basement Membrane Matrix (BD Biosciences)-coated chambers. The culture plate was incubated for 24 h, followed by staining with 0.1% crystal violet and counted under a light microscope.

2.9 | Western blot analysis

Total protein extracts were separated using 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Then, the membranes were blocked with 5% skimmed milk and probed overnight at 4°C with specific primary antibodies, followed by incubation with anti-rabbit IgG antibody (dilution ratio of 1:1000; 075-1506, SeeBio Biotechnology Company). The diluted primary antibodies for our study were as follows: c-Myc (E-AB-62131, Elabscience), AKT (E-AB-30465, Elabscience), p-AKT (E-AB-20802, Elabscience).

2.10 | ISH and immunohistochemistry (IHC)

The same paraffin-embedded sample was serially sectioned, one for ISH, the other IHC staining. Detection of SNHG17 in ESCC was performed using the SNHG17 ISH Kit (BOSTER Biological Technology Co. Ltd, MK10905-h) according to the protocol provided by the manufacturer. Briefly, paraffin-embedded slides were de-paraffinized by immersion in xylene (10 min twice) and then rehydrated through serial ethanol (100%, 95%, 80%, and 70%) for 5 min each followed by immersion in DEPC-treated PBS for 5 min. Then slides were incubated with pepsin for 20 min at 37°C and hybridized with the SNHG17 probe (200 nM) overnight at 37°C. The samples were incubated with blocking solution for 30 min, anti-digoxigenin (anti-DIG) reagent was applied for 60 min, and the probe signal was visualized with diaminobenzidine (DAB) solution (BOSTER).

IHC assay was performed as described previously. The paraffin-embedded sections were dewaxed and hydrated in a xylene bath and absolute ethanol solution. Primary antibodies used for the study were as follows: c-Myc (ab32795), Ki-67(bs-23103R). The ISH and IHC results were evaluated as follows: 0 (no staining, no positive cells), 1 (1%-25% positive cells), 2 (26%-50% positive), 3 (51%-75% positive) and 4 (>75% positive). Tissues with scores of 3 and 4 were classified as high expression, and those with scores of 0, 1, and 2 were deemed low expression. To eliminate the subjective evaluation error of percentage, ImageJ software (National Institutes of Health, USA) was used to score signals for inconsistencies in the percentages of the IHC and ISH signals.

2.11 | Isolation of cytoplasmic/nuclear RNA

The nuclear and cytoplasmic fractions of Eca109 and Kyse150 cells were isolated according to the protocol of the PARIS™ Kit Protein and RNA Isolation System (Invitrogen). Total RNA was isolated simultaneously from independent cytoplasmic and nuclear fractions. Finally, qRT-PCR was used to determine the relative expression of SNHG17 to estimate the relative ratios of SNHG17 in nuclear and cytoplasmic RNA concentrations.

2.12 | RNA-binding protein immunoprecipitation assay (RIP)

RIP was performed for Eca109 and Kyse150 cells using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore). Eca109 and Kyse150 cells were lysed in complete RIP lysis buffer (Millipore). The lysates were incubated with magnetic beads (Millipore) conjugated to anti-c-Jun antibody (Cell Signaling Technology) and control IgG antibody. Finally, total RNA was extracted for subsequent qRT-PCR analysis.
2.13 | RNA pull-down assay

Biotin-labeled RNA pull-down assays were performed on Eca109 cells as instructed by the supplier for Pierce™ Magnetic RNA-Protein Pull-Down Kit (Thermo Fisher Scientific). SNHG17 and antisense-SNHG17 were transcribed in vitro from linearized constructs using the RiboMAX™ Large-Scale RNA Production Systems (Promega). Streptavidin magnetic beads (Promega) were applied to isolate the RNA-binding protein complexes. Finally, proteins were detected by western blot assay.

2.14 | Chromatin immunoprecipitation (ChIP)

ChIP assay was conducted in Eca109 cells. Cross-linked chromatin DNA was sheared into fragments (200-1000 bp) using ultrasound, and immunoprecipitated with anti-c-Jun (Cell Signaling Technology). After washing the beads, purified DNA was isolated for qRT-PCR.

2.15 | Luciferase reporter assay

Different sequences of the c-Myc promoter region (~1000 bp to transcription start site [TSS]) containing or lacking a c-Jun-binding element were added into the pGL3-basic vector (Promega). Eca109 cells were transfected with pGL3-c-Myc (~998 to +73) or pGL3-Myc (~335 to +73) plasmids. Luciferase activity was assessed after 48 h using a Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase was used for normalization.

2.16 | Tumor formation assay

Here, G-418 bioreagent (Merck Co., Ltd) was used to generate stable Eca109 cells transfected with pcDNA3.1SNHG17 or empty vector. Here, 100 μL of the cell suspension (1 x 107 cells/mL) were subcutaneously injected into 1 side of BALB/nu nude mice (aged 4-6 wk; Beijing HFK Bioscience Co., Ltd). Tumor formation was examined every 4 d and the tumor volumes calculated. The mice were dissected after 30 d, and tumors were excised from mice for weight assessment. Animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Fourth Affiliated Hospital of Hebei Medical University.

2.17 | Statistical analysis

All statistical analyses were performed using the SPSS 22.0 software. Student t test or paired Student t test was conducted to compare between the 2 groups. Chi-square ($\chi^2$) test was applied to analyze the association between SNHG17 expression and clinicopathological features. All experiments data were presented as mean ± SD from 3 independent experiments performed in duplicate. $P < .05$ indicated statistical significance.

FIGURE 1 | SNHG17 is elevated in ESCC tissues and cell lines and associated with a poor prognosis. A, GEO database (GSE20347) analysis showed the expression of SNHG17 in 17 ESCC tissues and matched para-tumorous tissues. B, Expression level of SNHG17 in 182 ESCA tumor samples and 13 normal controls from TCGA database. C, Expression of SNHG17 in ESCC cell lines and pools, as shown by qRT-PCR. Pools: average expression in 10 normal tissues was used as the normal control. D, Expression of SNHG17 in ESCC tissues and matched para-normal tissues was detected by qRT-PCR. Heatmap shows the expression of SNHG17 in each pair of samples. E, Kaplan-Meier curves of SNHG17 in ESCC patients for OS. Error bars are shown as mean ± SD. *$P < .05$, **$P < .01$, ***$P < .001$
### Table 1: Correlation of the expression level of SNHG17 with clinicopathologic features of ESCC patients

| Characteristics          | SNHG17 expression |          |          |          |
|--------------------------|-------------------|----------|----------|----------|
|                          | N                 | Low n (%) | High n (%) | P-value  |
| Age (y)                  |                   |          |          |          |
| <58                      | 59                | 32 (54.29) | 27 (45.76) | .470     |
| ≥58                      | 69                | 33 (47.83) | 36 (52.17) |          |
| Gender                   |                   |          |          |          |
| Male                     | 72                | 39 (54.17) | 33 (45.83) | .385     |
| Female                   | 56                | 26 (46.43) | 30 (53.57) |          |
| TNM stage                |                   |          |          |          |
| I                        | 14                | 12 (85.71) | 2 (14.29)  | <.001*** |
| II                       | 42                | 34 (80.95) | 8 (19.05)  |          |
| III                      | 57                | 14 (24.56) | 43 (75.44) |          |
| IV                       | 15                | 5 (33.33)  | 10 (66.67) |          |
| Depth of invasion        |                   |          |          |          |
| T1                       | 17                | 14 (82.35) | 3 (17.65)  | <.001*** |
| T2                       | 19                | 16 (84.21) | 3 (15.79)  |          |
| T3                       | 78                | 31 (39.74) | 47 (60.26) |          |
| T4                       | 14                | 4 (28.57)  | 10 (71.43) |          |
| Pathological differentiation|             |          |          |          |
| Well/moderate            | 102               | 57 (55.88) | 45 (44.12) | .022*    |
| Poor                     | 26                | 8 (30.77)  | 18 (69.23) |          |
| Lymph node metastasis    |                   |          |          |          |
| Negative (N0)            | 63                | 41 (65.08) | 22 (34.92) | .01*     |
| Positive (N1/2/3)        | 65                | 24 (36.92) | 41 (63.08) |          |
| Distant metastasis       |                   |          |          |          |
| Negative                 | 102               | 51 (50.00) | 51 (50.00) | .726     |
| Positive                 | 26                | 14 (53.85) | 12 (46.15) |          |
| Family history of upper gastrointestinal cancer | | | | |
| Negative                 | 99                | 52 (52.53) | 47 (47.47) | .466     |
| Positive                 | 29                | 13 (44.83) | 16 (55.17) |          |
| Mortality                |                   |          |          |          |
| Survive                  | 57                | 36 (63.16) | 21 (36.84) | .012*    |
| Die                      | 71                | 29 (40.85) | 42 (59.15) |          |

*P < .05; **P < .01; ***P < .001.

### Table 2: Univariate and multivariate Cox regression analysis for clinicopathological features associated with prognosis of 128 ESCC patients

| Variables                                | Univariate analysis | Multivariate analysis |
|------------------------------------------|---------------------|-----------------------|
|                                          | HR (95% CI)         | P                     | HR (95% CI)         | P                     |
| Age (<58 vs ≥58)                         | 1.040 (0.654-1.656) | .867                  | 0.984 (0.601-1.612) | .950                  |
| Gender (male vs female)                  | 1.285 (0.800-2.065) | .299                  | 1.190 (0.718-1.973) | .183                  |
| Lymph node metastasis (negative vs positive) | 1.961 (1.226-3.138) | .005**                 | 1.796 (1.060-3.044) | .030*                 |
| Distant metastasis or recurrence (negative vs positive) | 2.243 (1.075-0.931) | .031*                  | 2.573 (1.144-5.784) | .022*                 |
| Pathological differentiation (well/moderate vs poor) | 1.303 (0.765-2.245) | .341                  | 1.532 (0.829-2.834) | .174                  |
| Depth of invasion (T1/2 vs T3/4)         | 1.226 (0.732-2.055) | .439                  | 0.647 (0.331-1.265) | .203                  |
| TNM stage (I + II vs III + IV)           | 2.352 (1.412-3.917) | .001**                | 1.998 (1.026-3.890) | .042*                 |
| Family history of upper gastrointestinal cancer (negative vs positive) | 1.158 (0.679-1.975) | .590                  | 1.496 (0.796-2.812) | .211                  |
| SNHG17 (high vs low)                     | 2.355 (1.456-3.809) | <.001***              | 1.925 (1.011-3.668) | .046*                 |

*P < .05; **P < .01; ***P < .001.
FIGURE 2 SNHG17 boosts the proliferation, lessens apoptosis, and elevates migration, invasion, and EMT of ESCC cells in vitro. A, Expression of SNHG17 was detected by qRT-PCR after transfection with shSNHG17 for 48 h in Eca109 and Kyse150 cells. B, C, MTS and colony forming assays were performed to evaluate Eca109 and Kyse150 cell proliferation after knocking down SNHG17. D, Apoptosis of ESCC cells was determined by flow cytometry apoptosis analysis under SNHG17 knockdown. E, Cell migration and invasion ability were analyzed by transwell assays (magnification: ×100). F, G, qRT-PCR and western blot were performed to examine the expression levels of E-cadherin and N-cadherin in ESCC cells. Error bars are shown as mean ± SD, **P < .01.
3 | RESULTS

3.1 | SNHG17 is elevated in ESCC tissues and cell lines and associated with a poor prognosis

Based on searches of GEO datasets (GSE20347) and GEPIA (http://gepia2.cancer-pku.cn/index), the analysis showed that SNHG17 was markedly upregulated in esophagus cancer samples (Figure 1A,B). For further analysis, primers were designed for different transcript variants of SNHG17 to identify the transcript of SNHG17 that boosted the expression of SNHG17 in ESCC cell lines. Transcript 7 (954 nt long, NR_152757.1) was expressed highly in 5 ESCC cell lines compared with a panel of human esophageal cancer cell lines (Figures 1C and S1). Moreover, 128 pairs of ESCC and adjacent normal tissues were evaluated. Patients were divided into 2 groups based on the median value: high SNHG17 expression (fold change ≥ median, n = 63) and low SNHG17 expression (fold change ≤ median, n = 65) groups. As shown in Figure 1D, SNHG17 expression in cancer tissues was higher than that in the adjacent normal tissues. Upregulated SNHG17 expression was related to TNM stage, depth of invasion, tumor differentiation, lymph node metastasis, and mortality (Table 1). Additionally, Kaplan-Meier survival analysis indicated that patients with highly expressed SNHG17 had a significantly poor overall survival (Figure 1E). Univariate and multivariate Cox regression analyses demonstrated that upregulated SNHG17 was an independent prognostic marker for ESCC patients (Table 2).

3.2 | SNHG17 boosts the proliferation, lessens apoptosis, and elevates migration, invasion, and EMT of ESCC cells

To address the biological functions of SNHG17 in ESCC cell lines, we transfected Eca109, Kyse150, and TE1 cells with shSNHG17 or overexpression vector. The transfection efficiency of SNHG17 was measured by qRT-PCR. As demonstrated in Figures 2A and S2, the expression of SNHG17 was significantly decreased by shSNHG17#1/#2 and markedly increased by the overexpression vector compared with the control groups. MTS and colony formation assays showed that the downregulated SNHG17 significantly attenuated cell viability and colony generation compared with the control group (Figure 2B,C). Moreover, flow cytometry cell apoptosis analysis indicated that depletion of SNHG17 increased the apoptosis of ESCC cells (Figure 2D). Transwell assay demonstrated that the migratory and invasive abilities of Eca109 and Kyse150 cells were drastically weakened after shSNHG17 transfection (Figure 2E). Conversely, overexpression of SNHG17 notably increased the proliferation, migration, and invasion of TE1, Eca109 and Kyse150 cells.

FIGURE 3 SNHG17 is upregulated in TGF-β-induced Eca109 cells and involved in TGF-β1-mediated EMT. A, Expression of SNHG17 was assessed in Eca109 treatment with TGF-β1. B, The hallmarks of EMT were detected in TGF-β1-treated Eca109 cells. C, Eca109 cells were transfected with siNC and siSNHG17 for 24 h. Then, cells were transfected again and treated with or without 10 ng/mL TGF-β at 6 h after transfection. Images were acquired at 48 h after the treatment, and representative images of spindle-like shape cells are shown. Red square areas are magnified in the right panel. D, E, Eca109 cells were transfected with siNC and siSNHG17 for 24 h. The cells were then transfected again and treated with or without 10 ng/mL TGF-β1 at 6 h after transfection. At 48 h after treatment, qRT-PCR, and western blot assessed the mRNA and protein expression of EMT-related markers, respectively. Data are presented as mean ± SD. **P < .01.
compared with vector-transfected cells (Figure S2). Additionally, qRT-PCR and western blot were performed to examine the EMT-related markers. The results exhibited that the level of E-cadherin expression was dramatically increased, while N-cadherin expression was significantly reduced in SNHG17-deficient Eca109 and Kyse150 cells (Figure 2F,G). These findings suggested that high expression of SNHG17 has a crucial role in facilitating the progression of ESCC.

3.3 | SNHG17 is upregulated in TGF-β1-induced Eca109 cells and involved in TGF-β1-mediated EMT

EMT is a key player in promoting cancer cell migration and invasion.25 Moreover, since TGF-β1 is required for EMT, we further estimated whether SNHG17 regulates the migration and invasion of ESCC cells via TGF-β1-mediated EMT. Strikingly, SNHG17 increased
its expression following TGFβ1 treatment in Eca109 cells (Figure 3A). The expression of Eca was significantly downregulated, while the levels of N-cadherin, Twist1, and SNAI2 expression in Eca109 cells was increased following TGF-β1 treatment (Figure 3B). As SNHG17 was induced by TGF-β1, we further investigated whether silencing SNHG17 abrogated TGF-β1-mediated EMT. As shown in Figure 3C, TGF-β1-induced EMT in Eca109 cells produced an epithelial-to-mesenchymal signature. The cells treated with TGF-β1 changed their original cobble-stone shape to a spindle-like shape, while depletion of SNHG17 prominently restrained the spindle-like formation. In addition, downregulation of SNHG17 largely overrode the TGF-β1 treatment suppressed E-cadherin and induced the expression of N-cadherin, Twist1, and SNAI2 in SNHG17-deficient cells (Figure 3D,E). Taken together, these data indicated that SNHG17 may be an EMT-related IncRNA and contribute to TGF-β1-induced EMT in ESCC cells.

3.4 | SNHG17 upregulates the expression of c-Myc and mediates the PI3K/AKT pathway in ESCC

To elucidate the potential mechanism by which SNHG17 promotes the progression of ESCC, we performed an RNA-sequencing assay
after Eca109 cells were transfected with shSNHG17. In total, 215 protein-coding genes were upregulated, while 145 were downregulated. Based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database pathway enrichment analysis and Gene Ontology terms of all essential genes, these data suggested that most genes in the PI3K/Akt signaling pathway were enriched and related to the proliferation and migration of cells (Figures 4A and S3). Among these, the top 5 genes were c-Myc, CLDN4, IL7R, JAK3, and AREG, and their
Figure 6  SNHG17 recruits c-Jun, which regulates the expression of c-Myc by binding to its promoter region. A, qRT-PCR for mRNA levels of c-Jun and c-Myc in the transfection of Eca109 and Kyse150 cells. B, Western blot assays for protein levels of c-Jun and c-Myc in transfected Eca109 and Kyse150 cells. C, Predicted c-Jun binding site in the −1000 bp region of the c-Myc promoter using JASPAR (E1: −17 to −30 bp; E2: −103 to −116 bp; and E3: −757 to −770 bp). D, ChiP-qPCR assays were conducted to show direct binding between c-Jun and c-Myc promoter regions in Eca109 (the P1 region contained E1 and E2 sites, the P2 region contained the E3 site). E, Verified c-Myc promoter sequences were added into a pGL3 vector. The activity of the c-Myc promoter containing or lacking c-Jun-binding elements was detected as luciferase reporter assays. F, G, Interaction between SNHG17, c-Jun, and c-Myc was confirmed by luciferase reporter assays. H, ChiP-qPCR analysis revealed that SNHG17 fortified the enrichment of c-Jun in the c-Myc promoter through the region of SNHG17 binding to c-Jun. IgG was the normalized control for qRT-PCR analysis. I, Expression of c-Myc was measured by qRT-PCR assay in SNHG17, SNHG17-MUT, or si c-Jun and SNHG17 co-transfected Eca109 and Kyse150 cells. J, qRT-PCR for mRNA levels of c-Myc in the transfection of Eca109 cells. K, L, qRT-PCR and western blot assays showed the expression of Twist1 in the different groups of Eca109 cells of overexpressing c-Myc/c-Myc + si c-Jun/c-Myc + si c-Jun + SNHG17. Data are shown as the mean ± SD, **P < .01, ***P < .001.

Regulation under SNHG17 silencing or overexpression was evaluated by qRT-PCR. The data showed that the level of c-Myc mRNA was decreased with knockdown of SNHG17, but increased with ectopic upregulation of SNHG17 (Figure 4B). Moreover, expression of c-Myc mRNA was positively correlated with SNHG17 expression in 128 cases of ESCC tissues (Figure 4C). Additionally, the expression of c-Myc, p-PI3K and p-AKT proteins was downregulated with the knockdown of SNHG17, whereas protein expression was dramatically upregulated with the ectopic expression of SNHG17 (Figure 4D). c-Myc was confirmed to having a prominent role in sustaining tumor growth in many tumor types, including ESCC. As shown by ISH and IHC, we found that tumor samples for high expression of SNHG17 showed an increased expression frequency of c-Myc protein, and positive staining occurred in the nucleus (Figure 4E). Therefore, we speculated that SNHG17 positively regulates c-Myc expression.

3.5  SNHG17 binds to transcription factor c-Jun

The dysregulation of SNHG17 decreased the expression of c-Myc dramatically. Furthermore, overexpression of SNHG17 increased the transcriptional activity of the c-Myc promoter, indicating that SNHG17 potentially regulated c-Myc by acting on its promoter (Figure 5A). Subsequently, to explore the molecular mechanism by which SNHG17 modulates c-Myc expression, we further examined the localization of SNHG17 through subcellular fractionation in Eca109 and Kyse150 cells, illustrating that SNHG17 was predominantly localized in the nucleus (Figure 5B). According to a previous study, lncRNAs located in the nucleus regulated the transcriptional process through binding to transcription factors (TFs), which in turn degenerated the DNA sequence motifs on the promoter in specific oncogenes in cancer cells. Therefore, we proposed a tentative mechanism in which SNHG17/TF may activate c-Myc expression through binding to the promoter region. First, we predicted the TF binding to the c-Myc promoter region using PROMO and University of California, Santa Cruz (UCSC) genome browser (Figure 5C). Then, we evaluated the possibility of SNHG17 interaction with the TFs via the catRAPID algorithm. Notably, the interaction between SNHG17 and c-Jun was higher (Figures 5D and S4A,B). CatRAPID fragments were used to estimate individual interaction propensities of polypeptide and nucleotide sequence fragments, further suggesting that the 100-400 nucleotide positions of the SNHG17 sequence may bind to the amino acid residues of the c-Jun protein with high propensivity (Figure 5E). c-Jun is also a TF that regulates TGF-β1 at the transcriptional level. We also found that depletion of c-Jun attenuated the ectopic expression of SNHG17 induced by TGF-β1 (Figure S4C). Therefore, TF c-Jun was selected for further research.

To evaluate the interaction between SNHG17 and c-Jun, both whole cell lysate and nuclear fraction were incubated with biotinylated SNHG17 or its antisense RNA transcribed in vitro, the results of the RNA pull-down assay revealed that SNHG17 bound to c-Jun mainly in the nucleus. Reciprocally, a c-Jun RIP assay was performed, and further showed that SNHG17 was enriched in the anti-c-Jun immunoprecipitation in Eca109 and Kyse150 cells (Figure 5F,G). To further map the specific region of SNHG17/c-Jun interaction, deletion mutants of SNHG17 were constructed. Deletion mapping analysis identified that the 100-400 nucleotide region was required for SNHG17 interaction with c-Jun. To further examine which motif of c-Jun interacts with SNHG17, a truncated c-Jun was applied according to the protein domain; this showed that His-tagged c-Jun-truncated mutant (ΔA243-331aa) was more critical for c-Jun-SNHG17 binding (Figure 5H,1). However, we also found the expression of c-Jun was not altered by SNHG17 knockdown, suggesting that SNHG17 does not participate in the regulation of c-Jun (Figure S4D).

3.6  SNHG17 recruits c-Jun, which regulates the expression of c-Myc by binding its promoter region

Next, we analyzed the effect of c-Jun on c-Myc in ESCC cells. First, we concluded that the knockdown of c-Jun reduced, while the overexpression induced, the level of c-Myc mRNA and protein (Figure 6A,B). Consistently, c-Myc overexpression partially reversed the inhibition of c-Jun knockdown on the proliferation, migration, and invasion of esophageal cancer cells (Figure 5J). JASPAR CORE database was used to predict the binding sites of c-Jun in the c-Myc promoter; 3 potential positions were identified (Figure 6C). To further explore whether c-Jun directly bound to the c-Myc promoter, we found by ChIP assay that c-Jun was enriched in the region of the c-Myc promoter (Figure 6D). Importantly, overexpression of c-Jun
SNHG17/c-Jun/c-Myc axis promotes ESCC cells growth and metastasis in vitro and in vivo. A, B, MTS and colony formation assays were performed to examine the effect of the SNHG17/c-Jun/c-Myc axis on cell proliferation ability of Eca109 and Kyse150 cells. C, Transwell assay was performed for evaluating the effect of the SNHG17/c-Jun/c-Myc axis on cell invasion and metastatic ability of Eca109 and Kyse150 cells in each group. D, E, Eca109 cells stably overexpressing SNHG17 were subcutaneously injected into nude mice and xenografts. The growth curve and final tumor weight of xenografts from mice in groups with different treatments (n = 8). F, qRT-PCR analysis showed that the expression of SNHG17 and c-Myc in xenograft tissues with overexpression of SNHG17. G, Western blot assays were performed to measure c-Myc, E-cadherin, N-cadherin, Twist1, SNAI2, and Ki67 expression levels in xenografts of mice with different treatments. H, IHC staining of c-Myc, N-cadherin, and Ki-67 in the xenografts of mice in the 2 groups. The results are shown as the mean ± SD. **P < .001
markedly accelerated the c-Myc promoter activity in Eca109 cells but only in the presence of the vector containing the c-Myc promoter E1/E2 (Figure 6E).

Based on the above data that SNHG17 could bind to c-Jun, we further verified whether SNHG17 recruits c-Jun to bind within the region of the c-Myc promoter. An SNHG17-truncated mutant (SNHG17-MUT) (∆101-400 bp) was created and co-transfected with the pGL3-c-Myc promoter, the results demonstrated that SNHG17-MUT or lack of c-Jun-binding motifs had no effect on the activity of c-Myc transcription, while SNHG17 fortified the transcription activity of the c-Myc promoter. The potentiation of SNHG17 upregulation on the activity of c-Myc transcription could be attenuated by a c-Jun reduction (Figure 6F,G). Moreover, ChIP assay further revealed that SNHG17 overexpression could increase the enrichment of c-Jun on the c-Myc promoter, while overexpression of SNHG17-MUT did not influence the binding ability of c-Jun to the c-Myc promoter (Figure 6H). In contrast with SNHG17 or SNHG17-MUT, transfection of pcDNA3.1-SNHG17 + si c-Jun significantly reduced the effect of SNHG17 on c-Myc expression. (Figure 6I). According to previous publications, Twist1 is a direct transcriptional target of c-Myc in cancer. To assess that the expression promoting effect of c-Myc on Twist1, Eca109 cells. Then, qRT-PCR and western blotting assays were conducted to reveal that the expression of Twist1 was upregulated by the plasmid in pcDNA3.1 c-Myc. Nevertheless, upregulation of Twist1 was restored under conditions of low c-Jun expression, overexpressing c-Myc. While, SNHG17 overexpression could partly rescue the negative effect of de-regulation of c-Jun on Twist1 expression (Figure 6J-L). Overall, SNHG17 recruited c-Jun to promote c-Myc transcription and Twist1 transcription, which resulted in ESCC EMT.

3.7 | SNHG17/c-Jun/c-Myc axis promotes ESCC cell growth and metastasis in vitro and in vivo

Rescue assays were performed to observe whether SNHG17 boosted ESCC progression through the regulation of c-Myc, and whether c-Jun reduced the effect of SNHG17 on the c-Myc expression. The results showed that transfection of pcDNA3.1-SNHG17 + si c-Jun + pcDNA3.1c-Myc could partially reverse the inhibition of si c-Jun + pcDNA3.1c-Myc on the proliferation, migration, and invasion of esophageal cancer cells (Figure 7A-C).

Next, we performed tumor xenograft experiments to further investigate the effects of SNHG17 on tumor growth in vivo. In total, $1 \times 10^7$ Eca109 cells with stable expression of SNHG17 or control cells were injected subcutaneously into BALB/c nude mice. The tumors formed by SNHG17-upregulated xenografts increased in nude mice compared with those in the control group, and the tumor volume and weight were monitored (Figure 7D,E). Also, qRT-PCR analysis showed that overexpression of SNHG17 increased the expression of SNHG17 and c-Myc in xenograft tissues (Figure 7F). To further elucidate the tumorigenic ability of SNHG17, the data of western blot indicated that the levels of c-Myc, N-cadherin, Twist1, SNAI2, and Ki-67 were enhanced by SNHG17 upregulation in xenografts (Figure 7G). Consistently, IHC assay showed that the xenograft tissues formed by SNHG17 upregulation showed higher positive staining for c-Myc, N-cadherin and Ki-67 than that in the control group (Figure 7H). Together, these findings suggested that SNHG17 facilitates ESCC tumor growth in vivo.

4 | DISCUSSION

In the present study, we confirmed that SNHG17 was upregulated in ESCC tissues and cell lines and was a poor prognosis factor for ESCC patients. Next, functional assays showed that SNHG17 promotes ESCC cell proliferation, migration, and invasion, and restricts cell apoptosis. Furthermore, SNHG17 was required for TGF-β-induced EMT; it directly triggered c-Myc expression by recruiting TF c-Jun in ESCC progression. These findings potentially provided a new perspective to boost treatment efficacy and improve prognosis in ESCC patients.

Reportedly, SNHG17 functions as a cancer-associated lncRNA are linked to diverse diseases. Previously, Xu et al explained that upregulated SNHG17 promotes the proliferation and migration in non–small-cell lung cancer. Wu et al showed that SNHG17 aggravates prostate cancer progression via regulation of the homolog SNORA71B. In ovarian cancer, SNHG17 was deemed as an oncogene, which regulated the expression of CDK6 by acting as a molecular sponge for miR-214-3p. Accumulating evidence showed that SNHG17 exerts its regulatory role predominantly in the cytoplasm. In the current study, we verified that SNHG17 was predominantly located in the nucleus, as revealed by the cellular fractionation assay. This finding was consistent with the results from the study by Zhang et al. In the nucleus, lncRNAs mainly participate in tumorigenesis and progression at the epigenetic and transcriptional level. Liu et al demonstrated that SNHG12 located in the nucleus and upregulates CDCA3 by stabilizing the transcription factor SP1. In addition, SNHG15 promoted colon cancer progression through binding and maintaining Slug stability. Here, we assumed that, in the nucleus, SNHG17 might act as a transcription co-activator regulating the target genes at the transcriptional level. Here, RIP and RNA-pulldown assays illustrated that SNHG17 is directly bound to c-Jun in ESCC cells, which was different from that in the previous studies on the molecular mechanism of SNHG17 regulation. Furthermore, the study showed that the aberrant activation of c-Jun played a key role in regulating cellular invasion. This phenomenon could be activated or restrained by various types of modifications, such as phosphorylation or glycosylation. Xu et al clarified that SNHG1 induced MMP2 transcription via boosting c-Jun protein phosphorylation. Furthermore, we assumed that SNHG17 upregulation stabilized the c-Jun protein. ChiP assays determined that SNHG17 synchronously recruited c-Jun to c-Myc promoter regions and repressed transcription.
EMT is characterized by epithelial cells acquiring the mesenchymal cell phenotype, which is widely implicated in tumor invasion and metastasis. TGF-β, a potent EMT-inducing cytokine, promotes cancer cell invasion and metastasis.38-40 Previous studies have indicated that TGF-β induced several lncRNAs, such as lncRNA-ELIT-1,41 IncRNA-smd7,42 IncRNA UCA1,43 IncRNA-ATB,44 IncRNA NKILA,45 MALAT1,46 and MEG3,47 which are crucial for carcinogenic processes orchestrated by TGF-β. lncRNA-ELIT-1, lncRNA-ATB, IncRNA NKILA, MALAT1, and MEG3 are involved in EMT. In the current study, SNHG17 expression was upregulated by TGF-β treatment in Eca109 cells, which created an effect on EMT, including E-cadherin and N-cadherin. Furthermore, we found that depletion of SNHG17 prominently restrained the original cobble-stone shape to a spindle-like shape, spindle-like formation in Eca109 cell lines, and its depletion also largely overrode the inhibition of E-cadherin and the induction of N-cadherin by TGF-β treatment. Therefore, SNHG17 was deemed to be an EMT-related lncRNA and positively contributed to TGF-β-induced EMT in ESCC cells.

C-Myc is a vital tumor promoter, and its expression can be controlled by lncRNAs at the transcriptional and post-transcriptional levels.26 In previous findings, lncRNA PVT1, linc-ROR, and lncRNA CCAT1-L aggravated tumorigenesis and participated in c-Myc-dependent EMT.49-50 Moreover, Bhovmick et al.51 highlighted that TGF-β rapidly activates RhoA to promote the EMT procession of breast cancer, indicating that TGF-β-mediated EMT was independent of SMAD signaling. Grille et al.52 showed that EMT was activated by AKT in the HSCC cell line SCC15. This study provided an insight into the oncogenic function of SNHG17, which is partially dependent on c-Myc to exert a carcinogenic effect through rescue experiments. Furthermore, TGF-β-induced SNHG17 boosts protein expression of c-Myc, p-PI3K, and p-AKT. In addition, previous studies have confirmed that Twist1 acted a direct transcriptional target of Myc in cancer cells, and Twist1 elicited metastasis of MYC related-hCC.29,53 In the present study, we indicated that SNHG17 acted as an EMT-related lncRNA through TGF-β regulation to promote the expression of c-Myc and Twist1. Overexpression of SNHG17 and knocking down c-Jun could partially reverse the inhibition of c-Jun knock down on the migration and invasion of esophageal cancer cells. Therefore, we assumed that SNHG17 aggravated EMT progression via the SNHG17/c-Jun/c-Myc/Twist1 axis in ESCC.

Taken together, the present study demonstrated that TGF-β1-induced SNHG17 accelerated malignancy in ESCC. Additionally, SNHG17 stimulated the EMT process and exerted the oncogenic function depending on c-Myc by recruiting c-Jun to enhance c-Myc-expression and Twist1. As an upstream TF of TGF-β1, c-Jun regulates TGF-β1 at the transcriptional level.28 This phenomenon indicated that SNHG17-recruited c-Jun triggers TGF-β1-induced SNHG17. Moreover, the data provided a novel therapeutic perspective by targeting oncogenic SNHG17 in ESCC. Strikingly, the molecular mechanism of SNHG17 is considered as the entry point for further screening the targets of drugs antagonistic in vivo and in vitro experiments for ESCC.

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DISCLOSURE
The authors declare no potential conflicts of interest.

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