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LncRNA H19 promotes epithelial mesenchymal transition and metastasis of esophageal cancer *via* STAT3/EZH2 axis

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ABSTRACT

Background: Long non-coding RNA H19 (lncRNA H19) has been widely reported in esophageal cancer (EC), and previous study had found that lncRNAH19 was up-regulated in EC and promoted cell proliferation and metastasis. However, the mechanism still needs further studied.

Methods: Levels of lncRNA H19 were analyzed by qRT-PCR in matched samples from 30 patients. Expression levels of lncRNA H19, let-7, STAT3 and EZH2 were additionally identified by qRT-PCR and western blotting in five EC cell lines. The effects of lncRNA H19 on cell proliferation, migration, invasion and apoptosis in cell lines were performed by MTT assay, colony formation assay, Transwell assay and flow cytometry *in vitro*, and tumor formation was detected by xenograft nude mice model *in vivo*. The expression level of STAT3, EZH2, β-catenin, and EMT and metastasis related molecules such as E-cadherin, N-cadherin, Snail-1 and MMP-9 was assessed by qRT-PCR and western blotting. Finally, luciferase reporter assay and RIP assay were used to verify the interaction between lncRNA H19 and let-7c, and their subsequent regulation of STAT3.

Results: Knockdown of lncRNA H19 repressed cell proliferation, migration and invasion as well as EMT and metastasis *via* STAT3-EZH2-β-catenin pathway, while lncRNA H19 regulated STAT3 negatively regulated let-7c in EC cell lines.

Conclusions: lncRNA H19 facilitates EMT and metastasis of EC through let-7c/STAT3/EZH2/β-catenin axis.

1. Introduction

Esophageal cancer (EC), derived from different parts of the esophagus, are classified into esophageal adenocarcinoma (EAC) and esophageal squamous cell carcinoma (ESCC) (Stoecklein et al., 2008). It is the eighth most common malignancy in the world with a very low five-year survival rate, leading to one hundred thousand cancer-related lethality all over the world deaths per year (Van Rensburg, 1982). Until now, lots of scientists endeavored themselves to explore the underlying biological mechanism of EC, but little progress has been made in the effects of long non-coding RNAs (lncRNAs) in the progression of EC especially lncRNA H19 (H19) (Dong et al., 2017; Chen et al., 2018; Gao et al., 2018; Jiao et al., 2016; Xu et al., 2018; Ma et al., 2018; Yan et al., 2017; Zheng et al., 2016). lncRNAs may regulate miRNAs which in turn regulate the expression of target genes in ESCC (Ma et al., 2018), however, the underlying mechanism among lncRNA especially H19, let-7, STAT3, and EZH2 remains elusive and deserves further investigation.

EZH2 (drosophila zeste gene enhancer human homolog 2) is a core catalytic element of PRC2 (Polycomb Inhibition Complex 2) which can exert a transcriptional inhibitory effect on target genes by acting *via* histone methylation (Januario et al., 2017). Studies of tumor etiology have shown that EZH2 plays an important role in promoting the malignant transformation of precancerous lesions into malignant cancer (Gardner et al., 2017). Increasingly, experimental data has demonstrated that the abnormal expression of EZH2 is related to the progression of various cancers (Benetatos et al., 2013). The high expression of EZH2 may promote the biological properties of tumor cells such as invasive ability and proliferation ability (Karakashev et al., 2018).

Accumulating evidence demonstrates that let-7 and H19 is highly relevant in BDL mouse livers and trophoblastic spheroid adhesion (Zhang et al., 2019; He et al., 2019). This study uses EZH2 as a starting point to explore the relationship among EZH2, STAT3 and H19, and provides a theoretical direction for the clinical treatment of EC.

In this study, we performed qRT-PCR and western blotting to find that H19, STAT3, and EZH2 were highly expressed in malignant EC cell lines. We demonstrated that knockdown of H19 could inhibit cell

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proliferation, EMT and metastasis of EC. Furthermore, we proved by both over-expression and knock-down experiments that EZH2 regulated SOX4 and β-catenin resulting in the elevated EMT and metastasis *in vivo* and *in vitro*. Additionally, we demonstrated that STAT3 overexpression could reverse the effects of H19 knockdown on the expression of STAT3, EZH2 and β-catenin. Finally, we demonstrated that H19 regulated STAT3 *via* sponging let-7c in EC cell lines.

2. Materials and methods

2.1. Human clinical samples, cell lines and cell culture

30 paired EC tissues with corresponding non-tumor control tissues were obtained from both male and female patients who visited the Second Xiangya Hospital of Central South University from July 2016 to September 2017. Patient consent was attained and this study was approved by the Ethics Committee of the Second Xiangya Hospital of Central South University. Exclusion criteria was applied for those who received preoperative radiotherapy and chemotherapy, or were positive for hepatitis B, HIV and/or syphilis. The collected tissue samples were immediately conserved in liquid nitrogen after washing with the sterile phosphate-buffered saline (PBS).

Human EC cells (Eca109, kyse410, kyse510, TE-1, TE-5), human normal esophageal epithelial cells (HEEC), and HEK293T cells were purchased from ATCC and cultured in DMEM (Dulbecco's Modified Eagle Media) medium supplemented with 10% FBS (Fatal Bovine Serum), 100 mg/ml streptomycin and penicillin. All cells were maintained in a humidified incubator with 5% CO_2 at 37 °C.

2.2. RNA interference and overexpression

The shRNA of lncRNA H19, EZH2, STAT3 or their respective negative control shRNA was synthesized and cloned into the shRNA vector pGPH1 (GenePharma, Shanghai). H19 was overexpressed using the pLncEXP vector. EZH2 and STAT3 were overexpressed using the pcDNA3.1 vector. Reconstructed vectors were purified, validated, and subsequently transfected into HEK293 T cells by Lipofectamine 3000 according to the manufacturer's guidelines.

2.3. Quantitative real-time PCR (qRT-PCR)

Total RNA from EC cell lines or cancer tissues was extracted by TRIzol reagent, and the first strand of cDNA was synthesized by reverse transcription reaction kit (Takara, Japan). Relative expression levels of H19, STAT3, SOX4, EZH2, let-7c and β-catenin were detected by qRT-PCR with GAPDH or U6 serving as the internal control for mRNA or lncRNA respectively. The forward and reverse primers of these genes were listed as follows: H19, forward primer 5′- TCCCAGAACCCACAA CATGA-3′, reverse primer 5′- TGATGTTGGGCTGATGAGGT-3′; STAT3, forward primer 5′- CAGCAGCTTGACACACGGTA-3′, reverse primer 5′-AAACACCAAAGTGGCATGTGA-3′; SOX4, forward primer 5′- AGCG ACAAGATCCCTTTCATTC -3′, reverse primer 5′- CGTTGCCGGACTTCA CCTT -3′; EZH2, forward primer 5′-AATCAGAGTACATGCGACTG AGA-3′, reverse primer 5′- GCTGTATCCTTCGCTGTTTCC-3′; β-catenin, forward primer 5′ – CCTATGCAGGGGTGGTCAAC-3′, reverse primer 5′-CGACCTGGAAAACGCCATCA-3′;

let-7c, forward primer 5′- GCCGAGTGAGGTAGTAGGTTGT-3′, reverse primer 5′- CTCAACTGGTGTCGTGGA-3′; U6, forward primer 5′-CTCGCTTCGGCAGCACA-3′, reverse primer 5′-AACGCTTCACGAATTT GCGT-3′; GAPDH, forward primer 5′- GGAGCGAGATCCCTCCAA AAT-3′, reverse primer 5′- GGCTGTTGTCATACTTCTCATGG-3′;

2.4. Western blotting

The total protein of EC cell lines or cancer tissues were extracted by RIPA and protease inhibitors and the cellular debris was removed by ultracentrifugation. 10 μg of protein was loaded and separated by denatured SDS-PAGE, and subsequently transferred to PVDF membrane. This was blocked by 5% non-fat milk in 1% TBST. Target proteins were detected with primary antibodies (Abcam) at 4 °C overnight: STAT3 (ab5073, 1:1000), SOX4 (ab80261, 1:1000), EZH2 (ab186006, 1:1000), β-catenin (ab16051, 1:1000), E-cadherin (ab15148, 1:1000), N-cadherin (ab18203, 1:1000), Snail-1 (ab82846, 1:1000), MMP-9 (ab73734, 1:1000) (Zhang et al., 2019). Finally, the relative protein expression level was analyzed by Image J software and calculated as the density ratio of the target protein to GAPDH (ab22555; internal control).

2.5. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Eca-109 and TE-5 cells were prepared according to standard protocol. 24 h after shRNA transfection, 10 μl of MTT solution per well was added to achieve a final concentration of 0.45 mg/ml. Plates were incubated at 37 °C for 3 h then subsequently solubilized using 100 μl of corresponding solubilization solution. Absorbance was read at 490 nm by a microplate reader and correlated for cell proliferation

2.6. Colony formation assay

Eca-109 and TE-5 were seeded into the six-well culture plates and transfected for 24 h when wells reached 60–70% confluency. A total of 1×10^2 cells were then incubated for 2 weeks at 37 °C. Cell colonies were washed twice with PBS. Colonies (≥50 cells) were examined using a surgical microscope. The efficiency of colony formation was determined as number of colonies/number of cells inoculated × 100%.

2.7. Flow cytometry

H19 shRNA or negative control shRNA(shNC) was transfected into Eca-109 or TE-5 cell lines respectively. The effects of H19 on cell apoptosis were analyzed by flow cytometry using the annexin V-PI apoptosis kit (Beyotime, China) according to protocol. PI can distinguish live cells from dead cells with the use of a counterstaining dye.

For cell cycle assay, the Eca-109 or TE-5 cell lines were detached by 1% trypsin and washed three times in PBS at 4 °C Cells were then fixed with 70% ethanol at 4° C for 30 min. then washed three times with PBS, 200 μl solution of PI (50 μg/ml) was added and incubated for 1 h at room temperature, with ribonuclease pre-treatment ensuring only DNA was stained by PI. Flow cytometry was performed at 605 nm on a single cell suspension to analyze the cell cycle

2.8. Transwell migration and invasion assay

Eca-109 or TE-5 cells were re-suspended in serum-free medium and seeded into the upper chamber (1×10^5 in 200 μl serum-free medium). After 24 h incubation, cells on the upper surface of the membrane were removed with a cotton swab. Cells which migrated through the membrane coated with Matrigel (BD Biosciences, USA) into the lower chamber (The migrated cells in the lower chamber), were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet (Beyotime, China). For the invaded cells, transwell membranes coated with Matrigel (BD Biosciences, USA) were used to assay cell invasion *in vitro*. Images were captured using an inverted microscope.

2.9. Xenograft model in nude mice

All animal experiments were approved by the Committee of the Second Xiangya Hospital of Central South University. Female Sprague-Dawley rats (4–6 weeks old, 200–300 mg) were purchased from Hunan SJA Laboratory Animal Co.; Ltd. and housed at room temperature (22 ± 1 °C) on a 12/12-hr light/dark cycle, with access to food and water ad libitum. The NC- or shRNA-transfected Eca-109 or TE-5 cell

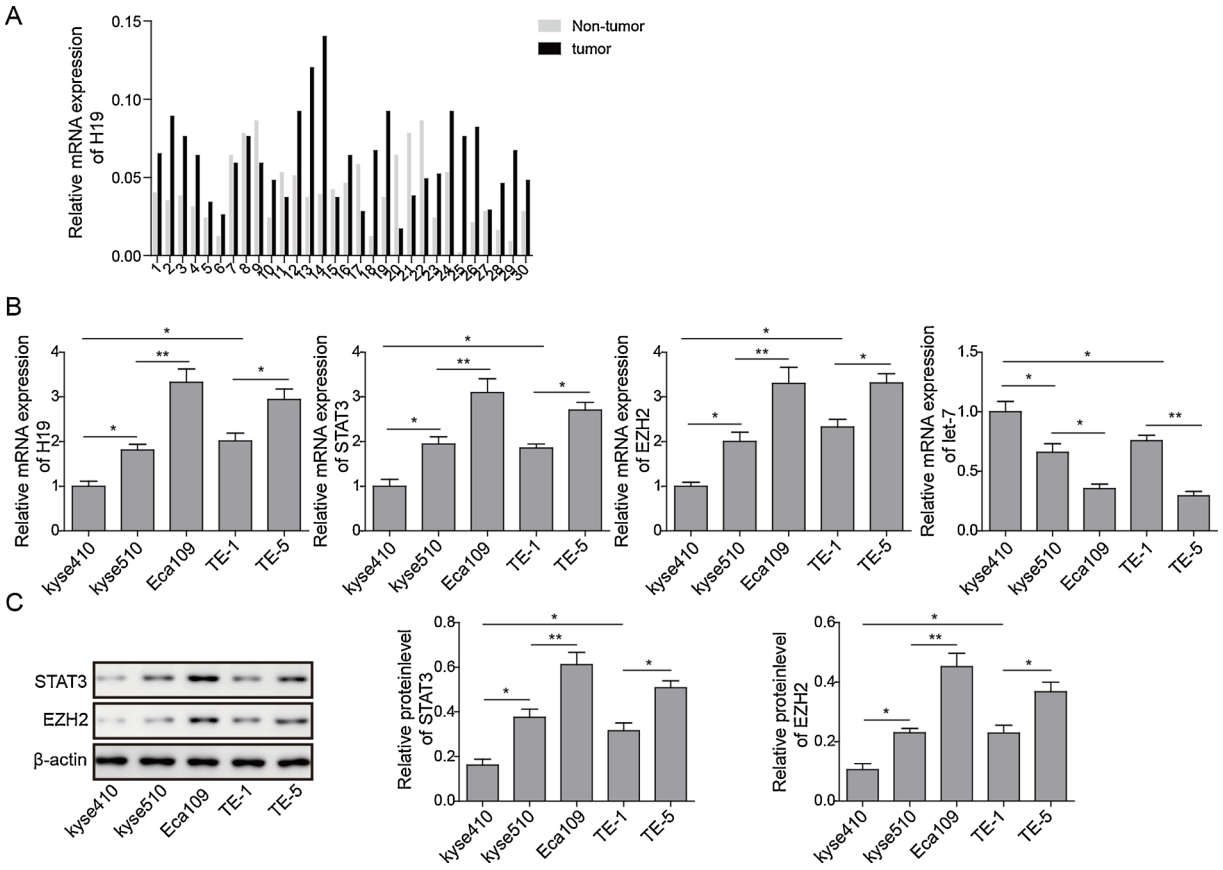

Fig. 1. The elevated expression level of H19 correlated with STAT3 and EZH2 in EC cells. (A) The mRNA expression level of H19 was evaluated by qRT-PCR in EC tissues as well as corresponding adjacent non-tumor tissues (n = 30, P < 0.05), (B) qRT-PCR showed higher expression of H19, STAT3, and EZH2 mRNA in Eca109, kyse410, TE-1, and TE-5, compared with HEEC. let-7 was significantly decreased in kyse410 and kyse510 when compared to Eca109 and also decreased in TE-5 when compared to TE-1. (C) Western blotting showed the protein expression level of STAT3 and EZH2 was higher in Eca109, kyse410, kyse510, TE-5 than HEEC cell lines. Each assay was performed for at least three biological replicates (*$P < 0.05$, **$P < 0.01$).

lines were grown in complete culture medium. Cells were collected by trypsin, washed, and resuspended as 100 µl aliquots each containing 3.0×10^6 cells for subcutaneous injection. The tumor diameter was measured using digital calipers weekly at weeks 1 to 4, and tumor volume was calculated using the formula: Volume = $\frac{\text{width}^2 \times \text{length}}{2}$.

2.10. H&E staining

Upon isolation, the EC tissues were fixed in 4% paraformaldehyde at 4 °C overnight and prepared into 4-µm paraffin-embedded sections. The tissues were then stained using hematoxylin and eosin (HE) staining kit (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

2.11. Immunohistochemistry (IHC)

Clinical samples were de-paraffinized and rehydrated as per routine protocol endogenous peroxidase was blocked by immersing slides in 3% hydrogen peroxide solution for 5 min and rinsing three times in TBS-20. TBS-20 was quickly removed to avoid drying, and covered with 3–4 drop of Ki-67 primary antibody. Slides were incubated for 1 h at room temperature then washed three more times with TBS-20. Biotin-conjugated second antibodies were immediately added and samples were incubated at room temperature for 30 min, washed, then subsequently 15 min in DAB solution. After counterstaining with Harris Hematoxylin and sealing the sections by coverslips, the sections were observed under microscope and analyzed by Image J.

2.12. Immunofluorescence (IF)

At 48 h post-transfection, TE-5 or Eca-109 cells were fixed with 4% PFA and permeabilized with 0.3% Triton ×-100 for 20 min. Cells were then blocked with 10% normal goat serum and incubated with anti-vimentin and anti-E-cadherin antibodies at 4 °C overnight. Cells were incubated with Alexa Fluor-488 or Alexa Fluor-555 conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h. Slides were mounted in VECTASHIELD Hardset with DAPI (Vector Laboratories, Burlingame, CA, USA). Images were acquired by LSM 710 NLO confocal laser scanning microscope.

2.13. Dual-luciferase reporter assay

Both wild type and mutant constructs of H19 3′UTR or STAT3 3′UTR were each cloned into pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, USA). HEK293 T cells were seeded in a 24-well plate and transfected with wild type or mutant H19/STAT3 3′UTR by lipofectamine 3000. Transfection and harvest efficiencies were controlled for using the pmirGLO reporter as an internal control. Cells were collected 48 h post-transfection and analyzed using the Dual-Luciferase Reporter Assay System (Promega, America).

2.14. RIP (RNA immunoprecipitation) assay

The Eca-109 or TE-5 cell lines were harvested and lysed by the NP40 lysis buffer (Sigma-Aldrich, America). Chromatin was sheared by ultrasound on ice. Co-precipitated RNA bound to primary AGO2 antibody

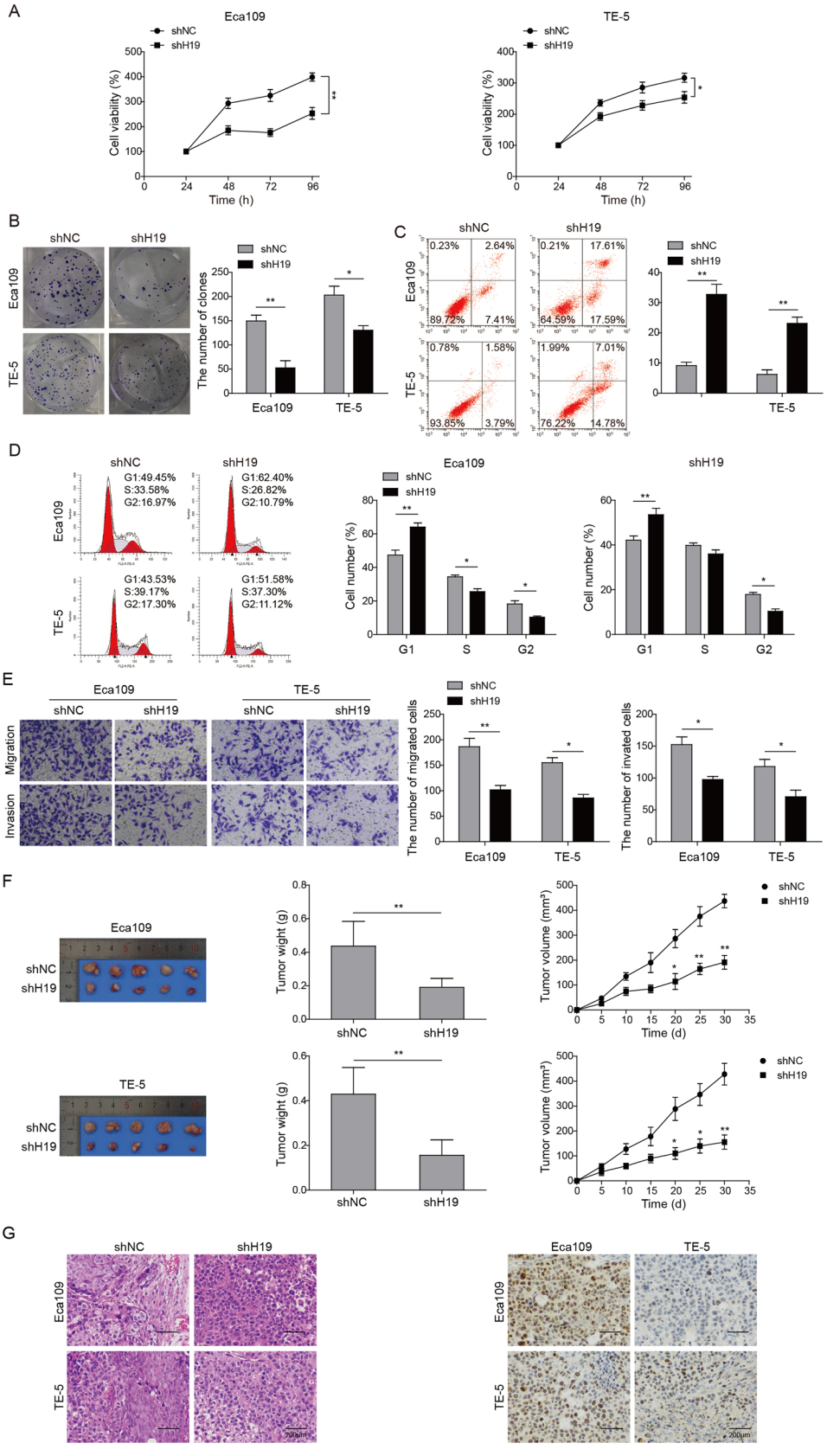

Fig. 2. Knockdown of H19 may inhibit cell proliferation, migration, invasion and promote apoptosis in EC. Eca109 and TE-5 cells were transfected with H19 shRNA. (A) Cell viability was determined by MTT assay. (B)The colony formation which represented as cell proliferation was performed by colony formation assay. (C) Cell apoptosis was evaluated by flow cytometry. Histogram indicated the apoptosis rates for EC cells. (D) Cell cycle distribution was performed by flow cytometry assay. Histogram indicated cell cycle distribution. (E) Migration and invasion was assessed after 24 h incubation by transwell assay. (F) The tumor volume and weight in xenograft nude mice model was detected by tumor formation in nude mice. (G) H& E staining for the pathological changes of EC and Ki67 IHC assay for tumor formation. Each assay was performed for at least three biological replicates ($*P < 0.05$, $**P < 0.01$).

was isolated and purified. cDNA was reverse transcribed from RNA and binding targets were analyzed by qRT-PCR.

2.15. Statistical analysis

All data was analyzed by SPSS 22.0 software and presented as mean ± SD. Student's *t* test was used to compare the mean values between two groups, and one-way analysis of variance (ANOVA) with Tukey's comparison analysis was performed among three or more groups. A P value of less than 0.05 was considered significant and a P value of less than 0.01 was considered statistically significant.

3. Results

3.1. Elevated expression level of H19 correlated with STAT3 and EZH2 in EC cells

In this study, we first determined the expression of H19 in 30 EC tissues and corresponding adjacent normal tissues by qRT-PCR. Our results showed that the mRNA level of H19 was significantly increased in EC versus normal tissue($P < 0.01$, Fig.1A). This result was validated in five EC cell lines (Eca109, kyse410, kyse510, TE-1, TE-5) and HEEC. Compared to HEEC, the level of H19 was elevated in all five PC cell lines, of which Eca109 and TE-5 had exhibited the most significant increase in H19 expression (Fig. 1B).

Consistent with previous studies, we reveal upregulation of STAT3 and EZH2 by H19 in all five cancerous cell lines relative to control. However, our data suggests a contrary pattern with regards to let-7. (Fig. 1B and Fig.1C). These results showed that up-regulation of H19 and down-regulation of let-7, which correlated with STAT3 and EZH2, maybe highly associated with EC development.

3.2. Knockdown of H19 may inhibit cell proliferation, migration as well as invasion and promote apoptosis in EC

We next studied the function of lncRNA H19 on proliferation, migration, invasion and apoptosis in Eca109 and TE-5 cell lines. These cell lines were stably transfected with H19 shRNA (shH19) or the negative control shRNA (shNC). MTT assay was performed to detect the effects of H19 on cell proliferation. Compared with control group, the cell proliferation was decreased in cells with sh-H19 (Fig.2A). Cells with H19 knockdown exhibited decreased colony formation (Fig.2B). Flow cytometry demonstrated that H19 knockdown led to more death by enhancement of apoptotic rate, and cells were arrested in phase G1 when compared to the control (Fig.2C and 2D). The transwell assay demonstrated that knockdown of H19 significantly inhibited migration and invasion (Fig. 2E). We further investigated the effect of H19 knockdown cell lines *in vivo* using a xenograft nude mice model, and confirmed that both esophageal tumor volume and weight were dramatically reduced. HE staining as well as Ki67 IHC assay further confirmed that H19 knockdown could reduce proliferation and tumor formation (Fig.2F and Fig.2G). These data suggest that knockdown of H19 may suppress cell proliferation, migration and invasion and promote cell apoptosis in EC cell lines.

3.3. EZH2 cooperates with STAT3 to regulate EMT and metastasis via SOX4-β-catenin pathway

Previous studies reported the importance of EZH2 in EMT and metastasis of various cancers. However, their potential relationship has never been fully explored in EC until now. In this study, the transwell assay was performed to demonstrate that Eca109 and TE-5 cells with EZH2 stable over-expression significantly aided migration and invasion, while knockdown of STAT3 attenuated this effect. Moreover, knockdown of EZH2 in Eca109 and TE-5 cells using shRNA could decrease the ability of migration and invasion, while STAT3 stable over-expression (STAT3-OE) could offset the reduction (Fig.3A and B). We additionally examined the localization and expression of epithelial marker E-cadherin and mesenchymal marker vimentin by immunofluoresence. As shown in Fig.3C, knockdown of EZH2 caused a significant induction of E-cadherin and an attenuation of vimentin in both EZH2-knockdown TE-5 cells, as demonstrated by well-organized cell-cell contact and polarity. This effect was partially contrary for in TE-5 cells with over-expression of STAT3 and knockdown of EZH2. Besides, over-expression of EZH2 (EZH2-OE) could reduce the E-cadherin and increase the vimentin, which was reversed in Eca109 cells with EZH2-OE and shSTAT3 (Fig.3C). The tumor formation in xenograft nude mice model showed the similar trend (Fig.3D and E). Finally, we evaluated the protein expression levels of key transcription factors in EMT and metastasis process such as E-cadherin, N-cadherin, Snail-1 and MMP-9 by western blotting assay. These results demonstrated EZH2-OE increased the expression levels of SOX4, β-catenin, N-cadherin, Snail-1 and MMP-9, while knockdown of STAT3 decreased the expression of E-cadherin. In addition, knockdown of EZH2 showed the opposite effects with EZH2-OE, which was counteracted by shEZH2 combined STAT3-OE (Fig. 3F and G). These results suggest that EZH2 synergistically with STAT3 may promote EMT and metastasis through SOX4-β-catenin pathway *in vivo* and *in vitro*.

3.4. STAT3 was regulated by lncRNA H19 in EC cells

To decipher the regulatory effects of H19 and STAT3 on EZH2/β-catenin pathway in esophageal cancer, Eca109 and TE-5 cancer cell lines were created to either overexpress H19 with knockdown of STAT3 or vice versa. The qRT-PCR and western blotting assay demonstrated that H19 over-expression could significantly increase the mRNA and protein level of STAT3, EZH2 and β-catenin while STAT3 shRNA could decrease the level to some extent. However, when H19 was knocked down, STAT3, EZH2 and β-catenin were remarkably decreased. This expression was restored by the overexpression of STAT3 in H19 knockdown cell lines (Fig.4A and B).The phenomenon revealed that H19 may regulate STAT3, as well as the expression of downstream molecules EZH2, SOX4 and β-catenin.

3.5. LncRNA H19 was directly targeted by let-7c in EC cells

Previous study had reported that H19 modulated let-7 availability by acting as a molecular sponge, and in another paper that H19 potentiated let-7 family expression through reducing PTBP1 binding to their precursors in cholestasis had been investigated (Kallen et al., 2013; Zhang et al., 2019). To further explore the regulatory effect of H19 and let-7c *via* STAT3, we performed dual luciferase assays to verify the specific binding of H19 and let-7c. In cell lines Eca109 and TE-5 with H19 knockdown, levels of let-7c were found to be decreased by qRT-PCR (Fig.5A). The dual luciferase assay revealed that let-7c was significantly suppressed in cell lines transfected with H19 wild type. This repression was partially rescued in cell lines expressing the mutated H19 construct (Fig.5B). Moreover, RIP assay was also adapted to further illustrate the specific proteins interacting with H19 and let-7c, In the let-7c/H19 group, the expression levels of H19 and let-7c immunoprecipitated with AGO2 were both decreased compared with the anti-normal IgG group (Fig.5C). The mRNA and protein level of STAT3 was repressed when Eca109 and TE-5 were transfected with let-7c mimic (Fig.5D). Furthermore, luciferase reporter assay demonstrated that co-transfection with let-7c mimic and sh-H19 further decreased the relative luciferase activity in wild type 3′-UTR of STAT3 but not in the corresponding mutant (Fig.5E).Thus, these results demonstrated that lncRNA H19 was directly targeted by let-7c and regulated STAT3.

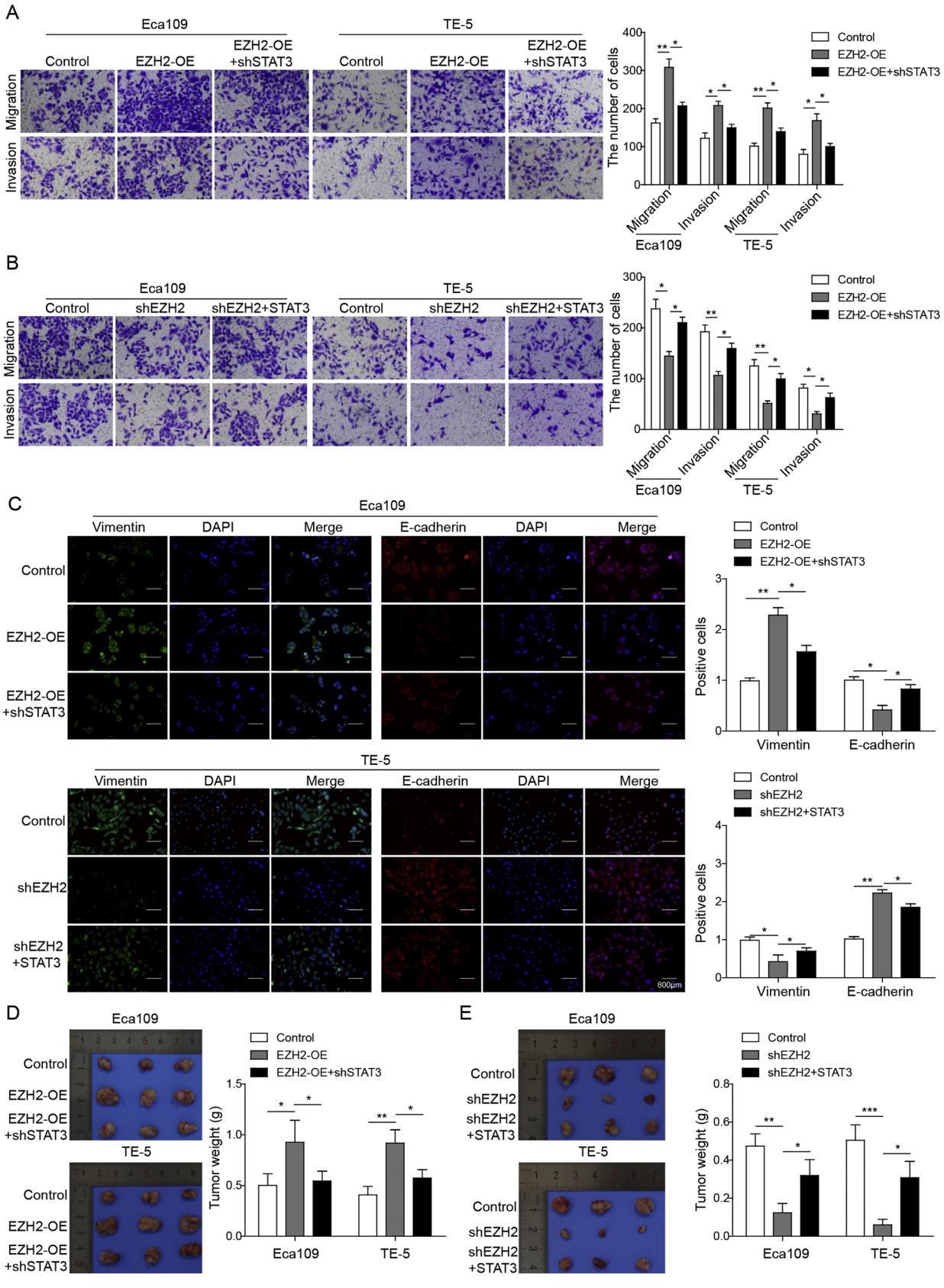

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Fig. 3. EZH2 cooperates with STAT3 to regulate EMT and metastasis *via* SOX4-β-catenin pathway. EZH2 was stably expressed with or without STAT3 knockdown in Eca109 and TE-5 cell lines. (A) Migration and invasion was assessed after 24 h incubation by transwell assay in cells with EZH2 stable expression with or without STAT3 knockdown. (B) Migration and invasion was assessed after 24 h incubation by transwell assay in cells with shEZH2 with or without STAT3 overpression. (C) Immunofluorescence was performed in cells with EZH2 stable expression with or without STAT3 knockdown, and cells with shEZH2 with or without STAT3 overpression (D) Xenograft nude mice model was applied for detection of tumor volume and weight in cells with EZH2 stable expression with or without STAT3 knockdown. (E) Xenograft nude mice model was applied for detection of tumor volume and weight in cells with shEZH2 with or without STAT3 overpression. (F)The protein expression level of STAT3, EZH2, β-catenin, E-cadherin, N-cadherin, Snail-1, MMP-9 was evaluated by western blotting in cells with EZH2 stable expression with or without STAT3 knockdown. (G) The protein expression level of STAT3, EZH2, β-catenin, E-cadherin, N-cadherin, Snail-1, MMP-9 was evaluated by western blotting assay in cells with shEZH2 with or without STAT3 overpression (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

4. Discussion

LncRNA are differentially expressed in various malignant human cancers, and some have proven to have diagnostic or prognostic value as biomarkers (Wang et al., 2018a, b; Ozes et al., 2017; Sun et al., 2018; Zhang et al., 2018; Cui et al., 2018; Peng et al., 2017; Zidan et al., 2018). Mounting evidence has confirmed that H19 is up-regulated in esophageal cancer and induces cell proliferation and metastasis (Tan et al., 2017). Nevertheless, underlying molecular mechanisms remain to be discerned.

In this study, we found that H19 was highly expressed in various EC cell lines and human cancer tissues. High expression of H19 was

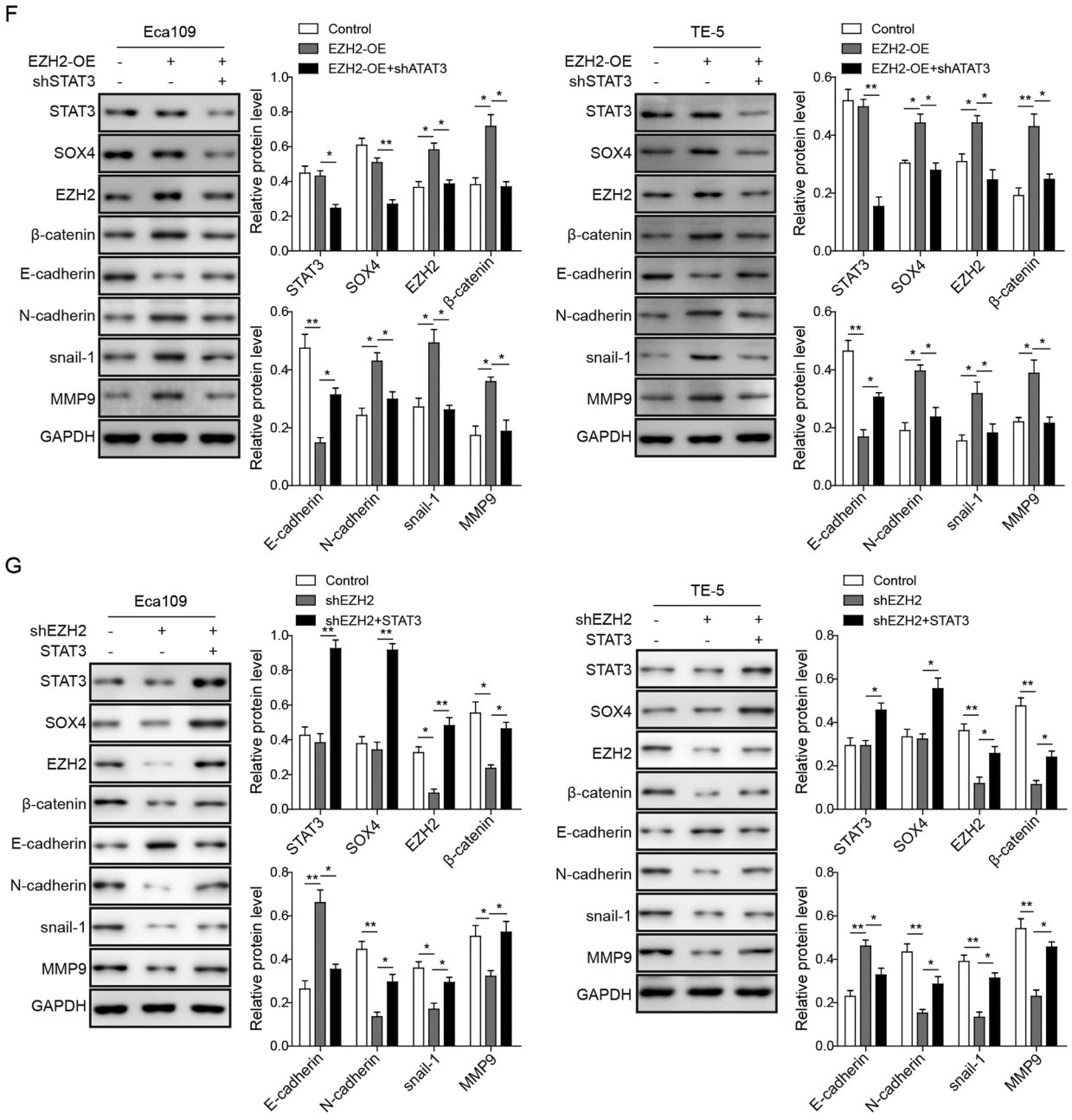

Fig. 3. (*continued*)

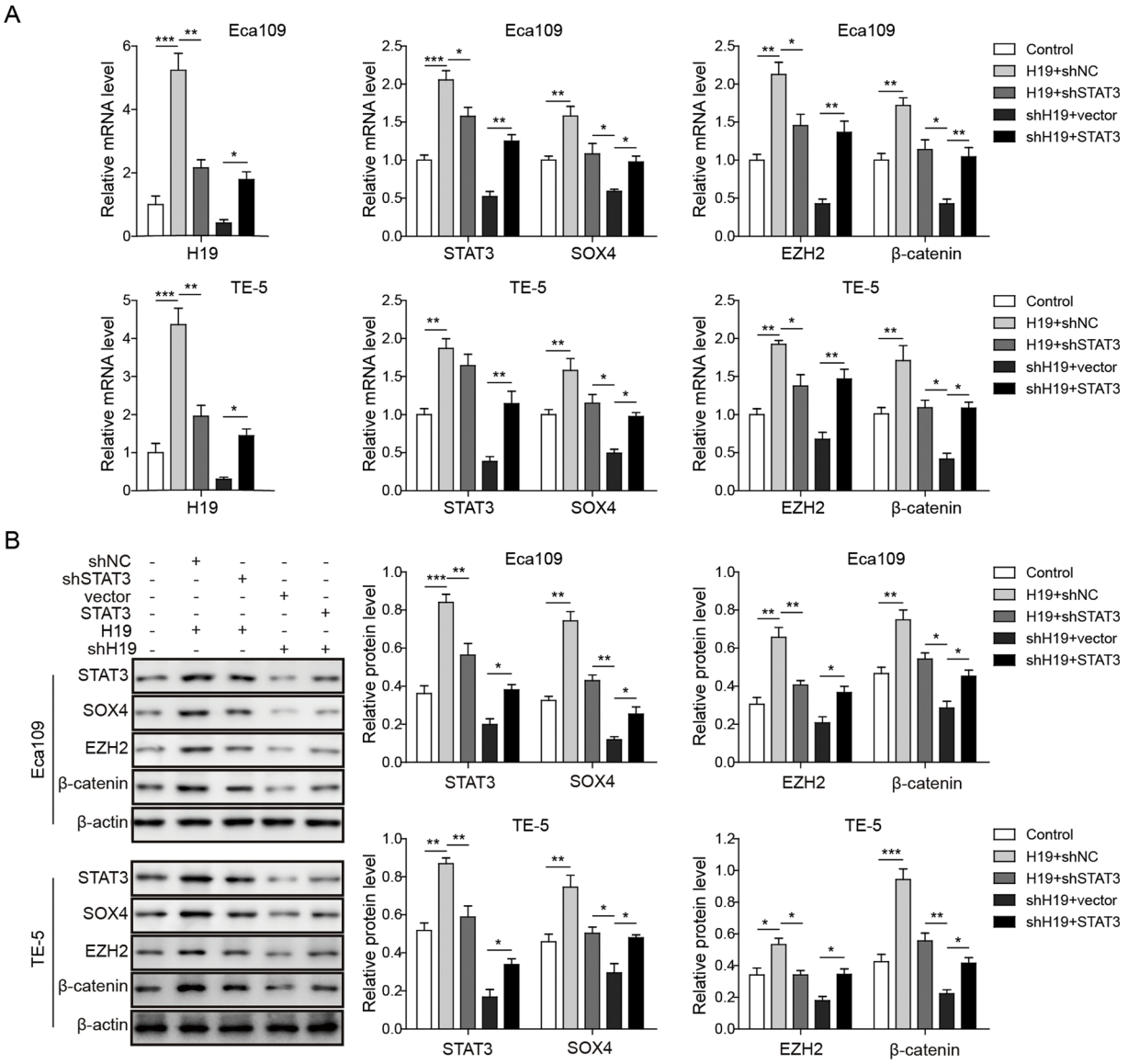

Fig. 4. STAT3 was regulated by lncRNA H19 in EC cells. (A) The mRNA expression level of H19, STAT3, SOX4, EZH2 and β-catenin was evaluated by qRT-PCR when EC cells was co-transfected with H19 stable expression and STAT3 shRNA, or co-transfected with STAT3 stable expression and H19 shRNA. (B) The protein expression level of H19, STAT3, SOX4, EZH2 and β-catenin was evaluated by western blotting when EC cells was co-transfected with H19 stable expression and STAT3 shRNA, or co-transfected with STAT3 stable expression and H19 shRNA. Each assay was performed for at least three biological replicates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

positively associated with increased expression of STAT3 and EZH2. Knockdown of H19 could inhibit proliferation and promote apoptosis in Eca109 and TE-5 cell lines. Furthermore, H19 was shown to inhibit EMT and metastasis *in vitro* and *in vivo*. EZH2 over-expression resulted in the increase of SOX4, EZH2 and β-catenin, then facilitated EMT and metastasis due to the increased expression of β-catenin, N-cadherin, Snail-1 and MMP-1 while EZH2 knockdown reversed these effects. What's more, knockdown of H19 could decrease the level of STAT3, SOX4, EZH2 and β-catenin. However, when STAT3 was over-expressed under this condition, the expression of STAT3, SOX4, EZH2 and β-catenin was reversed. Finally, we demonstrated that the inhibitory effect of let-7c on cancer cell self-renewal and tumorigenicity was exerted through STAT3, and was itself regulated by lncRNA H19 in two separate esophageal cancer cell lines, (Yu et al., 2007). This is supported by previous findings by Doki et al. whereby miRNA let-7 mediated cisplatin chemotherapy through IL-6/STAT3 signaling pathway in EC (Yu et al., 2007). Additionally, Lu et al. demonstrated that let-7 was lowly expressed in EC, repressed cancer cell proliferation and was highly associated with lymph node metastasis *via* negatively regulating HMGA2 (high mobility group A2) at the post-transcriptional level (Liu et al., 2014). Similarly, our results also showed the decreased expression of let-7 by qRT-PCR in EC cell lines such as kyse50, Eca109 and TE-5 when compared to the non-cancerous cells (Fig.1B). We further propose a novel mechanism by which let-7c interacts with H19 to the negatively regulate STAT3 in EC cells. This new mechanism suggestes that let-7 may serve as a therapeutic target in the treatment and diagnosis of EC and warrants further investigation at the clinical level.

In 2015, Andl CD et al. indicated that SOX4 forms a repressive complex with EZH2 and furthermore demonstrated that HDAC3 binds to the promoter chromatin of miR-31 and promotes the invasive EC progression and metastasis (Koumangoye et al., 2015), while the other possible molecular mechanisms of SOX4 and EZH2 in EC deserves much more attention. Canonical Wnt/β-catenin signaling pathway positively regulates various cancers including esophageal cancer *via* multiple regulatory networks and pave avenues to investigate potential strategies for invasive EC. Guo Q et al. recently reported that Msi2 played a

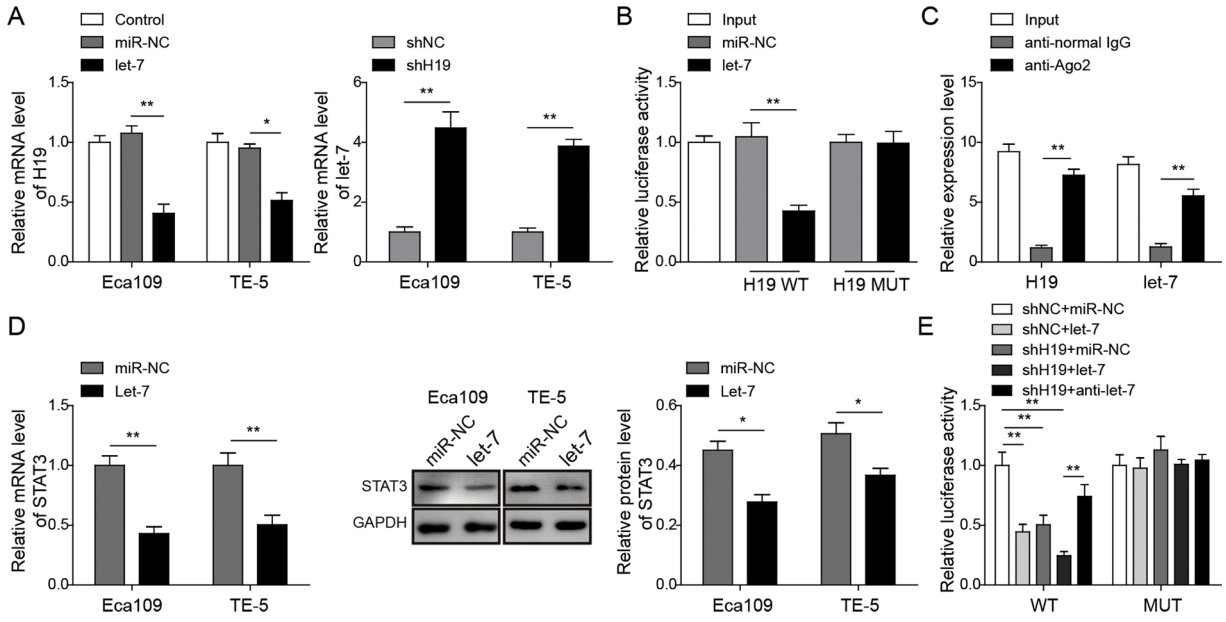

Fig. 5. LncRNA H19 was directly targeted by let-7c in EC cells. (A)The expression of H19 was determined by qRT-PCR in the EC cells transfected with miR-NC or let-7c, and the expression of let-7c was determined by qRT-PCR in the EC cells transfected with sh-NC or sh-H19. (B) HEK293 T cells were co-transfected with either let-7c mimic or NC and pmirGLO Dual-Luciferase miRNA Target Expression Vector with WT or Mut 3′-UTR of H19. Relative luciferase activity was determined by a dual-luciferase assay system. (C) RIP assay was performed using input, normal mouse IgG or anti-AGO2 primary antibodies. Relative expression levels of H19 and let-7c were detected by qRT-PCR. (D) The mRNA and protein expression of STAT3 was determined by qRT-PCR and western blotting in the EC cells transfected with miR-NC or let-7c. (E) The relative luciferase activity of H19 shRNA (shNC) and let-7c (miR-NC, anti-let-7c). Each assay was performed for at least three biological replicates ($*P < 0.05$, $**P < 0.01$).

positive role in ESCC through Wnt/β-catenin signaling and Hedgehog signaling pathways (Li et al., 2017). Additionally, Li et al. demonstrated that FZD7 could induce the nuclear translocation of β-catenin, activate the canonical Wnt/β-catenin and promote the development and metastasis of ESCC (Li et al., 2017). Furthermore, GOLPH3 and TNFAIP2 were also shown to regulate the tumorigenicity of EC *via* Wnt/β-catenin signaling (Wang et al., 2017a). However, the potential relationship between EZH2/SOX4 and β-catenin has never been fully explored until thus far. During the process of metastasis, SOX4 has been shown to exert its function in EMT by directly regulating the epigenetic enzyme EZH2, the poly comb group histone methyltransferase (Tiwari et al., 2013). Furthermore, it was demonstrated that the increased activity of EZH2 with corresponding STAT3 hyperphosphorylation served as marker for poor prognosis and low survival rate in gastric cancer. In gastric cancer, it was reported that STAT3 was hyper-phosphorylated and promoted the over-activation of EZH2, meanwhile STAT3 and EZH2 mediated the poor prognosis and low survival rate, therefore combination inhibition of STAT3 and EZH2 would be beneficial in the treatment of gastric cancer (Pan et al., 2016), and canonical Wnt/β-catenin/ZEB signaling pathway was well-known in cancer metastasis (Qu et al., 2015). In liver cancer metastasis, STAT3 facilitates the up-regulation of lncRNA HOXD-AS1 or lncSox4 by regulating SOX4 (Wang et al., 2017b). Herein, we have aimed to clarify the crosstalk between STAT3, EZH2 and β-catenin signaling pathways.

5. Conclusions

For the first time our results provide solid evidence that H19 could mediate the malignant metastasis of EC through STAT3/EZH2/β-catenin axis *in vitro* and *in vivo*. Moreover, we demonstrate that let-7 negatively regulates the function of H19 at the post-transcriptional level.

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Conflict of interest

The authors declare that they have no conflict of interest.

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