HPV E7 affects the function of cervical cancer cells via the TAL1/lnc-EBIC/KLHDC7B axis

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Abstract. High-risk human papillomavirus (HPV)16 and 18 are the primary cause of cervical cancer (CC) and long non-coding RNAs (lncRNAs/lncs) are often abnormally expressed in patients with CC. The authors' previous study indicated that oncogenic enhancer of zeste homolog 2 (EZH2)-binding lncRNA in cervical cancer (lnc-EBIC) serves a role in the tumorigenic activity of the HPV E6 protein in patients with CC. However, whether HPV E7 affects the development of CC through lnc-EBIC, and the potential mechanisms underlying this remains unclear. Therefore, the present study investigated the effects of lnc-EBIC and HPV E7 in cervical cancer cell lines HeLa, CaSki and C33A *in vitro*. CCK-8, EdU and DAPI staining assays, flow cytometry, RT-qPCR, western blotting and Transwell assay were performed on these cell lines. The results revealed that exogenous expression of HPV16/18 E7 significantly promoted lnc-EBIC expression, and conversely, lnc-EBIC was downregulated by silencing endogenous HPV16/18 E7 expression in corresponding CaSki and HeLa cells. Overexpression of lnc-EBIC significantly increased cellular proliferation, migration and invasion, and inhibited apoptosis in HPV$^-$ C33A cells. The tumorigenic effects of HPV16/18 E7 in corresponding CaSki and HeLa cells were significantly blocked by the silencing of lnc-EBIC expression. Molecular analysis revealed that HPV16/18 E7 depended on TAL BHLH transcription factor 1, erythroid differentiation factor inhibition to promote lnc-EBIC expression, which also resulted in the upregulation of oncogenic Kelch domain-containing 7B (KLHDC7B) in corresponding CaSki and HeLa cells. Additionally, KLHDC7B knockdown blocked the tumor-promotive effects of lnc-EBIC in HPV$^-$ C33A cells. Collectively, the results of the present study indicated that lnc-EBIC acts as an oncogenic lncRNA by enhancing KLHDC7B expression in HPV$^+$ and HPV$^-$ CC cells, and can be exploited by HPV16/18 E7 to accelerate tumorigenic activity in CC. These results further revealed that the lnc-EBIC/KLHDC7B axis represents a novel molecular mechanism and potential therapeutic target for CC.

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Introduction

High-risk human papillomaviruses (HPVs), particularly HPV16 and HPV18, are the main cause of cervical cancer, which ranks as the fourth most frequently diagnosed cancer and the fourth leading cause of cancer-associated deaths in women, worldwide (1). Additionally, the incidence and mortality rates of cervical cancer are increasing in several countries (2). HPV E6 and E7 are important viral oncogenes that act in concert to maintain the malignant phenotype of cervical cancer. These genes constitute attractive therapeutic targets, as it has been demonstrated that E6/E7 inhibition rapidly induces senescence in HPV-positive cancer cells (3). However, infection with oncogenic HPV alone is not sufficient for cancer development; genetic variations and epigenetic alterations are required for the development of precancerous lesions and cervical cancer (4,5). Thus, improving the clinical management of patients and developing an effective novel treatment strategy for patients with cervical cancer is a major challenge.

To determine the additional alterations that occur in cervical cancer, previous studies have demonstrated that E6 inactivates p53 by binding to the cellular ubiquitin ligase E6-associated protein, thereby preventing the replicative senescence of cervical cancer cells (6-8). As E6 contains a PDZ binding motif (PBM; including postsynaptic density

protein 95/DLG/zona occludins-1) at the extreme C terminus, it can further bind to a number of tumor suppressor protein-containing PDZ domains, including DLG, scribble and membrane associated guanylate kinase, WW and PDZ domain-containing 1 (9). In addition, the transforming activity of E7 is strongly increased when E6 is co-expressed; however, high-risk HPV E7 modulates the expression and degradation of several host proteins, including retinoblastoma (pRB), p107, p130, tyrosine-protein phosphatase non- receptor type14 (PTPN14) and p21, which leads to unscheduled cell cycle progression (10).

Recently, accumulating evidence has further confirmed that the modulation of long non-coding RNA (lncRNA/lnc) expression is an important aspect of oncogenic activity in high-risk HPV E6 and E7 proteins. For example, HPV 16 E6 increased the expression of lnc-cervical carcinoma expressed PCNA regulatory (CCEPR) and lnc-family with sequence similarity 83 member H antisense RNA 1 (FAM83H-AS1) through a mechanism that is not directly dependent on p53 inactivation, which thereby promoted proliferation and migration, and inhibited the apoptosis of cervical cancer cells (11,12). It has been previously demonstrated that HPV16/18 E6 and E7 proteins were associated with increased enhancer of zeste homolog 2 (EZH2)-binding lncRNA in cervical cancer (lnc-EBIC) expression in cervical cancer cells. The study additionally determined that E6 promoted lnc-EBIC expression to sequester certain tumor repressor microRNAs (miRs), including miR-375 and miR-139 that target HPV16/18 E6/E7 mRNA, thus forming a positive feedback loop that mutually derepressed gene expression in cervical cancer cells (13). Lnc-EBIC is a pseudogene that is highly expressed in human cervical cancer tissues and cell lines, which interacts with EZH2 to repress the expression of E-cadherin and thus promote cellular proliferation and invasion (14,15). In addition to E6, a previous study determined that HPV18 E7 also stimulated lnc-EBIC expression in HeLa cells (13). However, the tumorigenic roles and potential molecular mechanism of the E7/lnc-EBIC axis on cervical cancer has not been fully elucidated.

Transcriptome sequencing was performed in a previous study to analyze the effects of lnc-EBIC depletion on the mRNA levels of certain protein-coding genes (13). Oncogenic Kelch domain-containing 7B (KLHDC7B) was identified to be correlated with lnc-EBIC expression, and was determined to interact with Kelch-containing proteins via the C-terminal Kelch domain (16,17). Moreover, KLHDC7B was identified to be upregulated in breast cancer and could promote breast tumorigenesis by modulating genes involved in the interferon signaling pathway (16,18). In addition, recent transcriptome analysis further suggested that KLHDC7B could be used as a biomarker for prognostic prediction and may be involved in the development and progression of cervical cancer (19).

In the present study, the relationship between HPV16/18 E7 and lnc-EBIC in cervical cancer cells was investigated, and the effects of lnc-EBIC on the proliferation (using CCK-8 and EdU/DAPI staining assay), apoptosis [utilizing flow cytometer Annexin V/propidium iodide (PI) assay], cell invasion and migration (using Transwell assays) of HPV^+ and HPV^- cervical cancer cells were assessed, to provide a novel mechanism and potential therapeutic target for cervical cancer.

Materials and methods

Cell culture and transfection. Human cervical cancer cell lines, including HeLa ($HPV18^+$), CaSki ($HPV16^+$) and C33A ($HPV16^-/18^-$), were purchased from The Cell Bank of Type Culture Collection of the Chinese Academy of Sciences. Cells were cultured in DMEM-low glucose (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 μg/ml streptomycin (Beyotime Institute of Biotechnology), in a humidified atmosphere at 37˚C with 5% CO_2. Cells in the exponential phase of growth were used in the experiments. The protein-coding plasmids pCMV-Tag2B-HPV18 E7 and pCMV-Tag2B-HPV16 E7 were previously described (13). The nucleotide sequences of lnc-EBIC were synthesized and inserted into pcDNA3.1 vectors (Thermo Fisher Scientific, Inc.) to construct the pcDNA3.1-lnc-EBIC overexpression plasmids. Empty vectors were used as negative controls (NCs). A total of 4 μg of each plasmid vector was added to 100 μl of serum-free medium, and the volume of 100 μl of each mixture was added to $1x10^5$/ml cells in 6-cell culture plates. TAL1 small interfering (si)RNA duplexes were designed and synthesized at concentration of 100 nM (Guangzhou RiboBio Co., Ltd.). The sequences are as follows: siHPV18-E7, 5'-CCT TCTATGTCACGAGCAA-3'; siHPV16-E7, 5'-CACCTACAT TGCATGAATA-3'; silnc-EBIC, 5'-GGGAGTAAAGACTCC AGTA-3'; siTAL1, 5'-AACCATGGAATCAACAAGGAT-3'; siKLHDC7B, 5'-CAGTGACAATGACTGGGATAGTGCT-3'; siRNA NC, 5'-GTTCTCCGAACGTGTCACGT-3'. Plasmids were transiently transfected into cells using Lipofectamine® 2000 as instructed by the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.) at 37˚C for 48 h. After 48 h transfection, the selective overexpression and silencing of HPV16 E7, HPV18 E7, lnc-EBIC and TAL1 were detected by reverse transcription-quantitative (RT-q) PCR and western blotting.

RT-qPCR analysis. Total RNA (1 μg) was extracted from cells using the TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed into cDNA using an RT-PCR kit (Thermo Fisher Scientific, Inc.) in accordance with the manufacturer's protocol. qPCR was performed using SYBR Green (Takara Biotechnology Co., Ltd.) according to the manufacturer's instructions. The following primers were utilized: HPV16E7 forward, 5'-AGCAGAACCGGACAG AGCCCA-3' and reverse, 5'-TGTACGCACAACCGAAGC GT-3'; HPV18E7 forward, 5'-TGAAATTCCGGTTGACCT TC-3' and reverse, 5'-TCGGGCTGGTAAATGTTGAT-3'; lnc-EBIC forward, 5'-AAGG GCGTCGTGGTTCCAACTC-3' and reverse, 5'-AGCATTGCCGTCCTGGGTGTAG-3'; TAL1 forward, 5'-CAACTGGAAAATCCAAAGGCTATGG-3' and reverse, 5'-GACGCAATTCCTCCACAGTACACAG-3'; KLHDC7B forward, 5'-TGGGAACGAACACTCTTAC-3' and reverse, 5'-CAGCAACTGAACACTTGAC-3'. A total of 20 μl reaction mixture contained 1.5 μl of cDNA, 10 μl of 2x SYBR Primer Ex TagII (TaKaRa), 7.5 μl of ddH_2O and 1 μl of primers (10 μM). The ABI 7500 system (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used to perform the amplification reaction, using the following thermal cycling profile: 94˚C for 10 min, followed by 40 cycles of amplification (94˚C

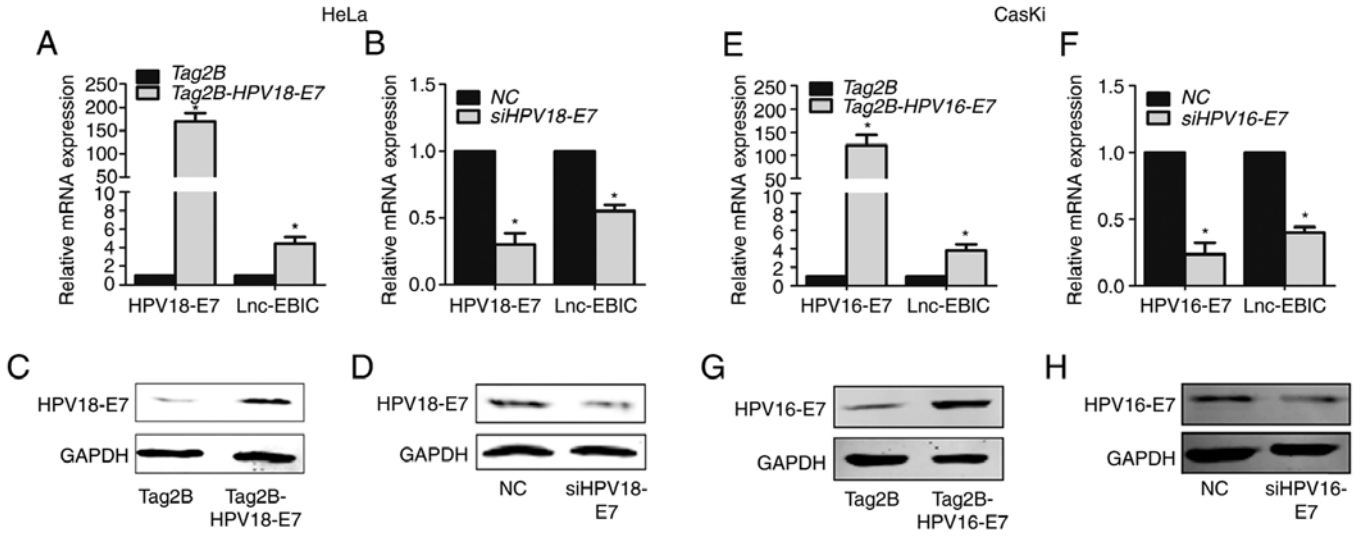

Figure 1. HPV16/18 E7 promotes the expression of lnc-EBIC. (A) Overexpression of HPV18 E7 enhanced the expression of lnc-EBIC in HeLa cells. (B) Depletion of HPV18 E7 reduced the expression of lnc-EBIC in HeLa cells. mRNA levels of HPV18 E7 and lnc-EBIC were detected by RT-qPCR. (C and D) The protein levels of HPV18 E7 were detected in HeLa cells; western blotting was performed with whole cell extracts of HeLa cells transfected with Tag2B-HPV18-E7 and siHPV18-E7. (E) Expression of lnc-EBIC was upregulated when the HPV16 E7 was overexpressed in CasKi cells. (F) Depletion of HPV16 E7 reduced the expression of lnc-EBIC in HeLa cells. mRNA levels of HPV16 E7 and lnc-EBIC were detected by RT-qPCR. (G and H) The protein levels of HPV16 E7 were detected in CasKi cells. Western blotting was performed with whole cell extracts of CasKi cells transfected with Tag2B-HPV16-E7 and siHPV16-E7. GAPDH was the loading control. Data are presented as the means ± SD; n=3. *P<0.05. HPV, human papillomavirus; lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; RT-qPCR, reverse transcription-quantitative PCR; si, small interfering; NC, negative control.

for 30 sec, 56˚C for 30 sec and 72˚C for 30 sec), and 72˚C for 10 min. Each experiment was performed in triplicate and was analyzed using the $2^{-\Delta\Delta Cq}$ method (20).

Western blot analysis. RIPA buffer (cat. no. P0013C; Beyotime Institute of Biotechnology) was used for the extraction and concentration determination of total protein from cells. Protein concentrations were determined with a BCA Protein Assay kit. Protein samples (30 μg) were separated via SDS-PAGE (8-10%) and transferred onto polyvinylidene fluoride membranes (EMD Millipore). The membranes were blocked using 5% non-fat milk for 2 h at room temperature, and then incubated with the following primary antibodies at 4˚C overnight: HPV16 E7 (cat. no. sc-6981; 1:1,000; 21 kDa) and HPV18 E7 (cat. no. sc-365035; 1:1,000; 15 kDa) both from Santa Cruz Biotechnology, lnc., p21 (product code ab109520; 1:1,000; 21 kDa), caspase-3 (product code ab32351; 1:5,000; 32 kDa), Bcl-2 (product code ab32124; 1:1,000; 26 kDa), c-JUN (product code ab32137; 1:5,000; 36 kDa), cysteine-rich 61 (Cyr61; product code ab24448; 1:1,000; 42 kDa), myosin regulatory light polypeptide 9 (MYL9; product code ab191393; 1:1,000; 20 kDa) and GAPDH (product code ab9485; 1:2,500; 37 kDa) all from Abcam. The membranes were subsequently incubated with HRP-conjugated IgG secondary antibodies (product code ab7090; 1:4,000; Abcam) at room temperature for 2 h. The chemiluminescence intensity was detected using an ECL kit (EMD Millipore) according to the manufacturer's protocol and ImageJ v1.8.0 software (National Institutes of Health) was used to analyze the gray value of the target band.

Cell Counting Kit-8 (CCK-8) assay. Cells were seeded into 96-well plates (Corning, Inc.) at a density of $1x10^4$/100 μl and incubated at 37˚C with 5% CO_2 for 24 h. CCK-8 (10 μl/ml; Dojindo Molecular Technologies, Inc.) was subsequently added to each well and incubated at 37˚C with 5% CO_2 for a further 4 h, after which the absorbance was measured using a microplate reader (Bio-Rad Laboratories, Inc.) at 450 nm.

Flow cytometric assay. The apoptosis of HeLa, CaSki and C33A cells was detected via flow cytometry. After transfection for 48 h, cells were collected and stained using an Annexin V-FITC/propidium iodide (PI) Apoptosis Detection kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Fluorescence signals were detected using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo 7.6.5 software (FlowJo LLC).

EDU and DAPI staining assay. Cellular proliferation and apoptosis was assessed using the BeyoClick™ EdU Cell Proliferation kit with Alexa Fluor 488 (Beyotime Institute of Biotechnology) and DAPI dihydrochloride (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. A total of $1x10^4$ cells were seeded in 24-well plates and incubated at 37˚C and 5% CO_2 with 10% FBS-medium for 12 h. Cells were then fixed in 4% paraformaldehyde for 15 min and permeabilized with 0.3% Triton X-100 for 20 min at room temperature. Cells were washed thrice with PBS and cultured at room temperature with 100 μl Click Reaction Mixture (50 μM) for 20 min in the dark. Cell nuclei were counterstained with 100 μl DAPI (1 mg/ml) at room temperature for 5 min. A fluorescence microscope of 10x20 (Carl Zeiss AG) was used to count the number of proliferative/apoptotic cells in three random fields of view per slide.

Transwell assay. For the cell invasion assay, the upper chamber (8-μm pore size; Costar; Coring, Inc.) was supplemented with Matrigel, while an upper chamber without Matrigel was used for the migration assay. Cells ($5x10^4$) were

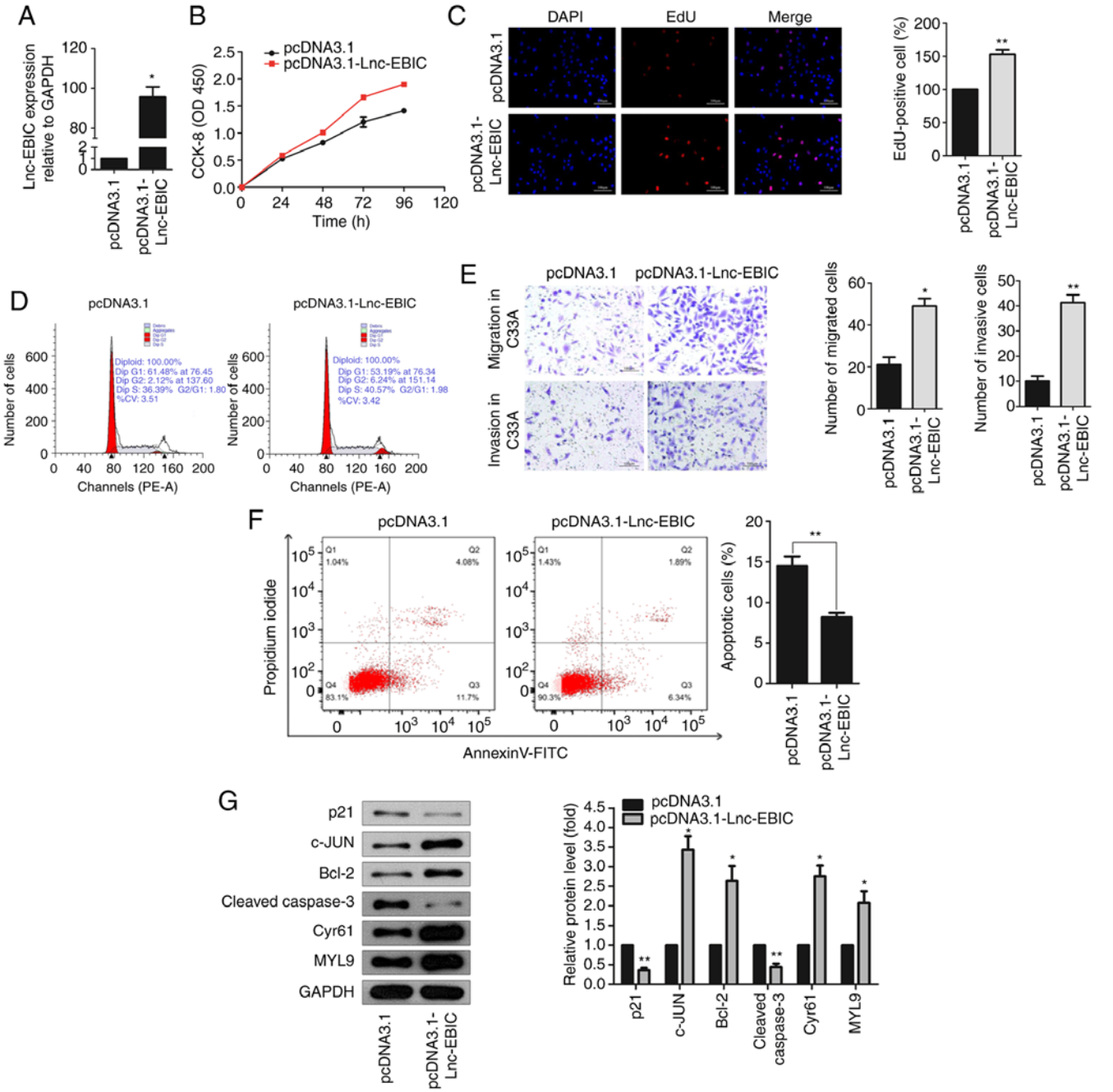

Figure 2. lnc-EBIC affects the function of cervical cancer cells. (A) The expression of lnc-EBIC in C33A cells was detected by RT-qPCR. C33A cells were transfected with pcDNA3.1 and pcDNA3.1-lnc-EBIC, respectively. (B) The proliferation of C33A cells was increased following lnc-EBIC overexpression. CCK-8 assays were performed at 24 h intervals as indicated. (C) The fractions of S-phase C33A cells were increased upon lnc-EBIC-overexpression. EdU assays were applied to visualize cells in the S-phase of the cell cycle. (D) Cell cycle analysis of the lnc-EBIC-overexpressed C33A cells. PI-stained C33A cells were subjected to FACS. (E) Transwell assays indicate that lnc-EBIC-overexpression increased C33A cell migration and invasion. (F) Overexpression of lnc-EBIC decreased the percentage of apoptotic cells. The percentage of cells in each quadrant is indicated. (G) The western blot analysis of cellular functional proteins on cell cycle, apoptosis, migration lysates from lnc-EBIC-overexpressed in C33A cells. The blots revealed lnc-EBIC increased the expression of c-JUN, Bcl-2, Cyr61, MYL9 and reduced the expression of p21 and Cleaved caspase-3. Data are presented as the means ± SD. n=3. *P<0.05 and **P<0.01. lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; RT-qPCR, reverse transcription-quantitative PCR; CCK-8, Cell Counting Kit-8.

resuspended in serum-free medium and plated into the upper chamber. Complete medium in the lower chamber was used as a chemical attractant. After incubation at 37˚C with 5% CO_2 for 48 h, the migrated or invasive cells attached to the lower chamber surface were fixed with 4% formaldehyde at room temperature for 15 min and stained with 0.5% crystal violet at room temperature for 30 min. The invasive or migrated cells in three random fields of view were subsequently imaged under an inverted light microscope. Experiments were performed independently and in triplicate.

Bioinformatics analysis. To determine the potential transcription factors that regulate lnc-EBIC expression, the promoter sequence of lnc-EBIC was extracted from the UCSC Genome

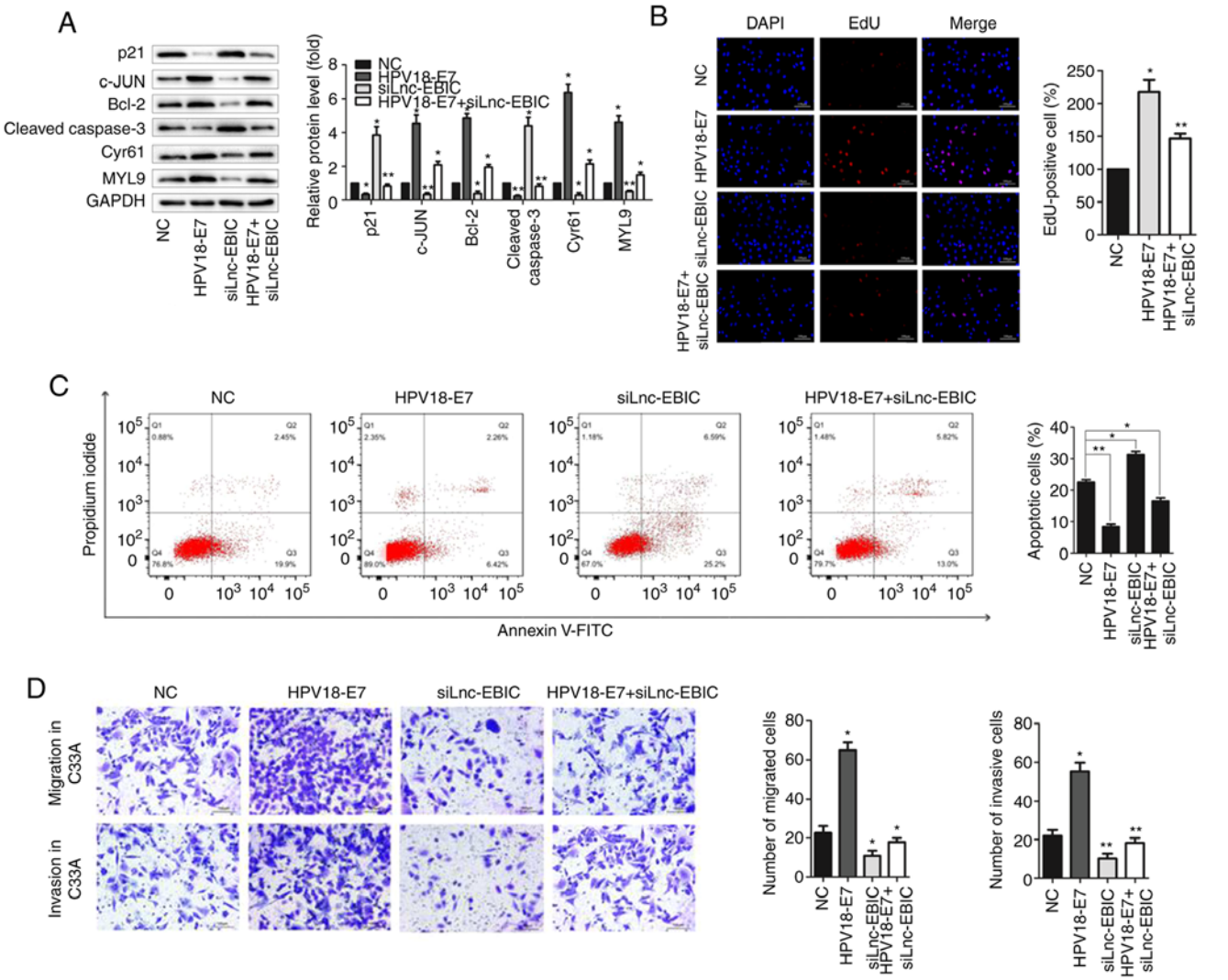

Figure 3. HPV E7 affects the function of Hela cells by regulating lnc-EBIC. (A) The western blot analysis of cellular functional proteins on cell cycle, apoptosis, migration lysates from HeLa cells. (B) The fraction of S-phase HPV18 E7 overexpressed HeLa cells was increased with co-transfection of HPV18 E7 and silnc-EBIC. (C) Interference of lnc-EBIC attenuated the effect of HPV E7 on apoptosis in HeLa cells. The percentage of cells in each quadrant is indicated. (D) Transwell assays indicated that interference of lnc-EBIC attenuated the effect of HPV E7 on migration and invasion in HeLa cells. Data are presented as the means ± SD, n=3. *P<0.05 and **P<0.01. HPV, human papillomavirus; lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; si, small interfering; NC, negative control.

Browser bioinformatics program (http://www.genome.ucsc.edu) (21) and analyzed via the Gene Transcription Regulation Database (GTRD; http://gtrd.biouml.org) (22), JASPAR (http://jaspar.genereg.net/) (23) and ChIP-Atlas-Enrichment Analysis program (http://chip-atlas.org) (24).

Statistical analysis. Data are presented as the mean ± SD and analyzed using GraphPad Prism V 6.00 software (GraphPad, Inc.). Statistical significance was determined using a paired Student's t-test or ANOVA followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

HPV16/18 E7 promotes the expression of lnc-EBIC. To determine whether lnc-EBIC is involved in cervical cancer progression, Tag2B-HPV18-E7 and Tag2B-HPV16-E7 were transfected into HeLa and CasKi cells, respectively. As presented in Fig. 1, the protein expression of E7 was increased in HeLa (Fig. 1A and C) and CasKi cells (Fig. 1E and G). Additionally, the expression of lnc-EBIC was significantly increased (Fig. 1A and E). To further confirm whether HPV16/18 E7 regulated the expression of lnc-EBIC, siRNA specific to HPV18 E7 and HPV16 E7 was transfected into HeLa and CasKi cells to knockdown endogenous E7 expression. The interfering efficiency of HPV16/18 E7 is presented in Fig. 1D and H. The results of RT-qPCR demonstrated that HPV16/18 E7 silencing significantly blocked the expression of lnc-EBIC (Fig. 1B and F). The results indicated that the HPV16/18 E7 protein promoted the excessive expression of lnc-EBIC in cervical cancer cells.

lnc-EBIC overexpression regulates the proliferation, apoptosis, the cell cycle, migration and invasion of HPV- C33A cervical cancer cells. To investigate the role of lnc-EBIC in cervical cancer, the pcDNA3.1-lnc-EBIC overexpression plasmid and corresponding NC were transfected into

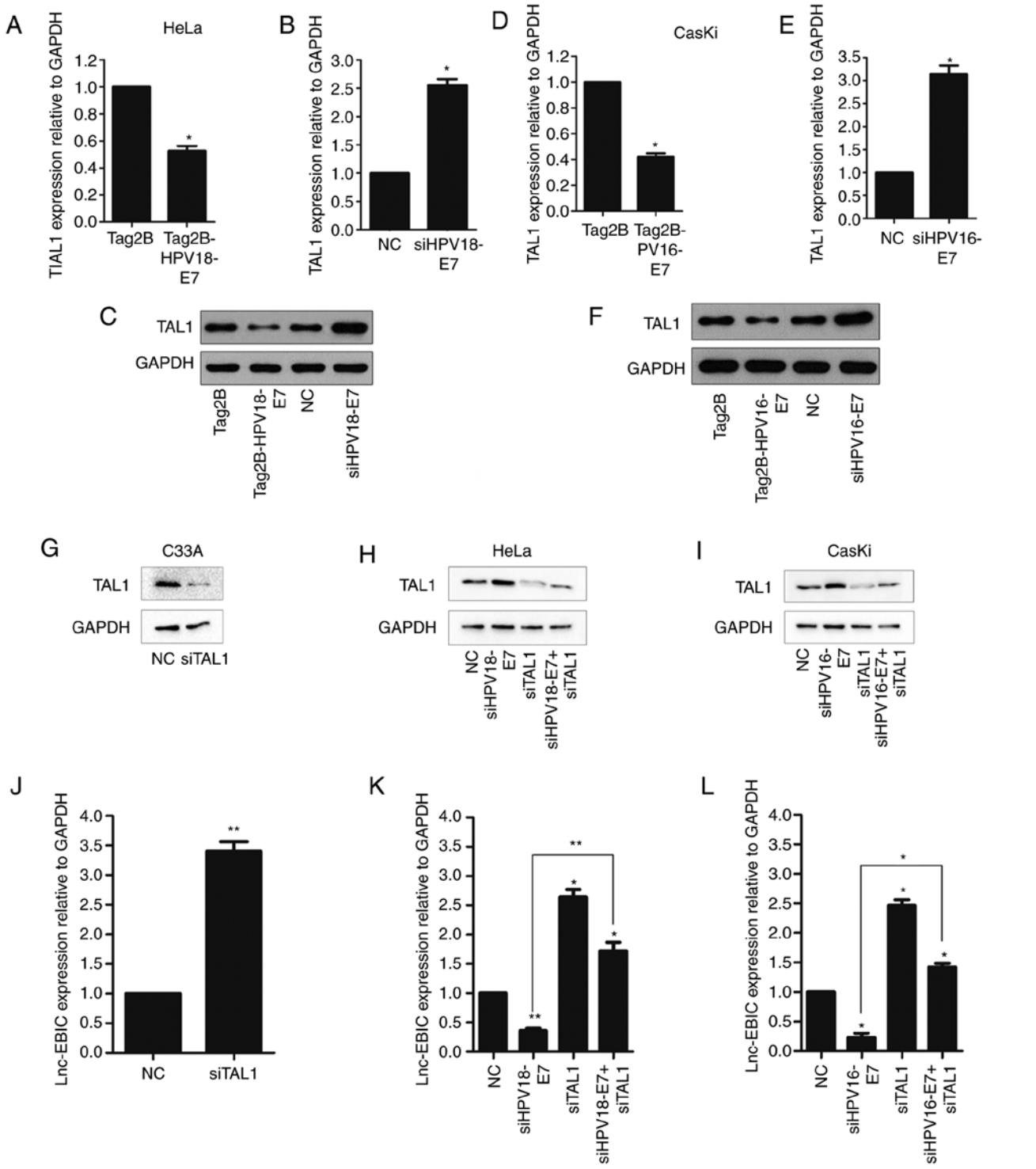

Figure 4. HPV E7 promotes the expression lnc-EBIC by inhibiting TAL1. (A and B) The overexpression and silencing of HPV18 E7 altered the expression of TAL1 in HeLa cells. (C) The protein levels of TAL1 were decreased following the overexpression of HPV18 E7 in HeLa cells and increased following the silencing of HPV18 E7 in HeLa cells. (D and E) The overexpression and silencing of HPV16 E7 altered the expression of TAL1 in CasKi cells. (F) The protein levels of TAL1 were decreased following the overexpression and increased following the silencing of HPV16 E7 in CasKi cells. (G) The protein levels of TAL1 were downregulated following the knockdown of TAL1 in C33A cells. (H and I) The protein levels of TAL1 were detected in HeLa and CasKi cells. (J) The mRNA levels of lnc-EBIC were upregulated following the knockdown of TAL1 in C33A cells. (K and L) TAL1 altered the effect of HPV E7 on lnc-EBIC in HeLa and CasKi cells. mRNA levels of TAL1 were detected by RT-qPCR. The protein level of TAL1 was detected by western blotting. GAPDH was used as the protein loading control. Data are presented as the means ± SD, n=3. *P<0.05 and **P<0.01. HPV, human papillomavirus; lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; TAL1, TAL BHLH transcription factor 1, erythroid differentiation factor; si, small interfering; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

HPV^{-} C33A cells (Fig. 2A). The results of the CCK-8 assay revealed that cell viability was significantly increased in C33A cells transfected with pcDNA3.1-lnc-EBIC compared with pcDNA3.1-transfected cells after 72 h (Fig. 2B). Additionally, EdU staining further confirmed that the upregulation of lnc-EBIC enhanced the proliferation of C33A cells (Fig. 2C).

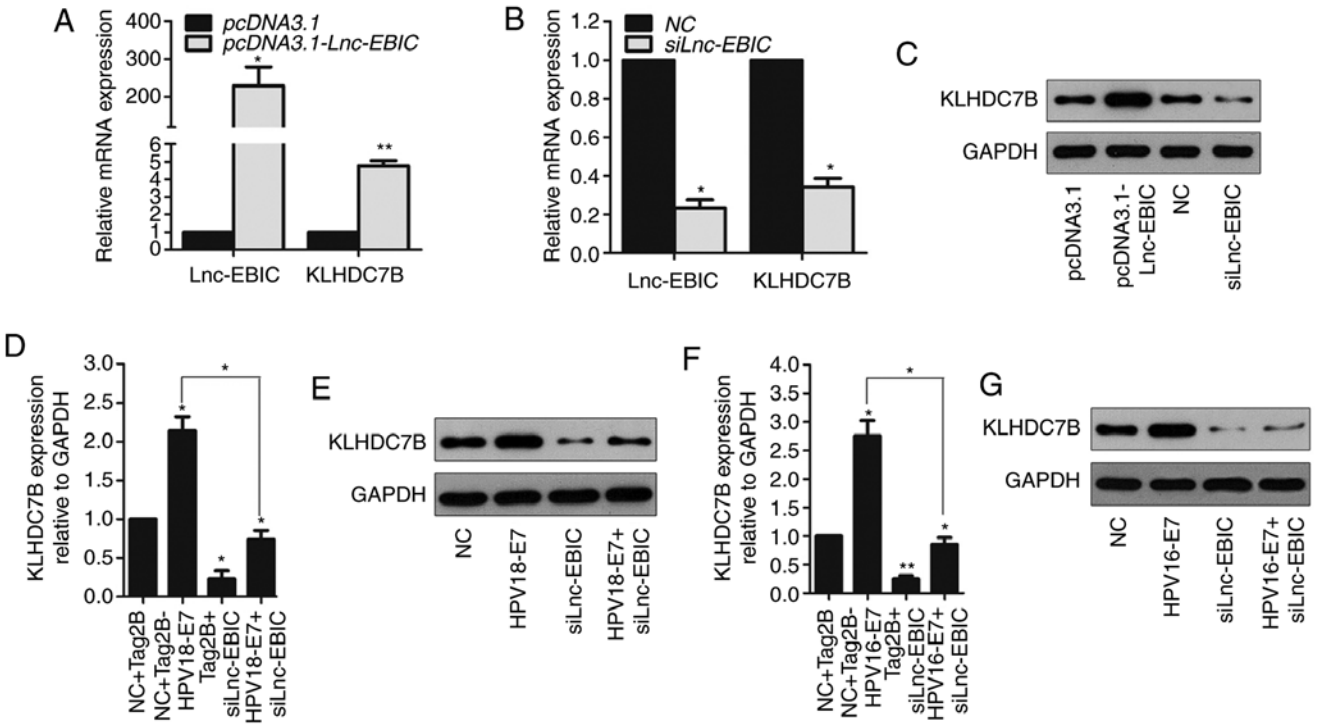

Figure 5. lnc-EBIC regulates the expression of KLHDC7B in C33A cells. (A and B) lnc-EBIC could positively regulate the mRNA levels of KLHDC7B in C33A cells. (C) Western blot analysis indicated that overexpression of lnc-EBIC boosted the protein levels of KLHDC7B and knockdown of lnc-EBIC suppressed the protein levels of KLHDC7B in C33A cells. GAPDH was used as the protein loading control. (D and E) HPV18 E7 influenced the mRNA and protein levels of KLHDC7B by regulating lnc-EBIC in C33A cells. (F and G) HPV16 E7 influenced the mRNA and protein levels of KLHDC7B by regulating lnc-EBIC in C33A cells. The mRNA levels of lnc-EBIC and KLHDC7B were detected by RT-qPCR. The protein level of KLHDC7B was detected by western blotting. GAPDH was used as the protein loading control. Data are presented as the means ± SD, n=3. *P<0.05 and **P<0.01. lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; KLHDC7B, Kelch domain-containing 7B; HPV, human papillomavirus; si, small interfering; NC, negative control; RT-qPCR, reverse transcription-quantitative PCR.

The effect of this upregulation on the cell cycle was then assessed via flow cytometry. The results demonstrated a markedly increased number of cells in the S and G2 phases (Fig. 2D), indicating that the upregulation of lnc-EBIC enhanced the cell cycle transition of cervical cancer cells. The results of the Transwell assay further demonstrated that cell migration and invasion were markedly increased in C33A cells transfected with pcDNA3.1-lnc-EBIC (Fig. 2E). In addition, an Annexin V-FITC/PI assay was performed to evaluate the apoptosis of C33A cells, the results of which revealed that the apoptotic rate was significantly reduced in lnc-EBIC-overexpression cells compared with the control (Fig. 2F). Moreover, the protein expression of pro-apoptotic p21 and Cleaved caspase-3 were decreased, and anti-apoptotic Bcl-2, c-JUN, Cyr61 and MYL9 were increased in lnc-EBIC-overexpressing C33A cells compared with pcDNA3.1-transfected C33A cells (Fig. 2G). The results indicated that lnc-EBIC promoted cell proliferation, cell cycle progression, migration and invasion, and inhibited the apoptosis of cervical cancer cells in HPV^{-} cervical cancer cells.

lnc-EBIC is required for the HPV16/18 protein, E7, to serve tumorigenic activities in cervical cancer cells. To determine the function of lnc-EBIC in E7-mediated tumorigenesis, siRNA targeting lnc-EBIC was co-transfected with an E7-overexpression plasmid in HeLa and CasKi cells (Fig. S1A and B). Western blot analysis revealed that lnc-EBIC knockdown significantly inhibited the promotive effects of E7 on anti-apoptotic proteins c-JUN, Bcl-2, Cyr61 and MYL9 in HPV^{+} cervical cancer cells, and increased the expression of pro-apoptotic proteins p21 and Cleaved caspase-3, which were decreased by E7 overexpression (Figs. 3A and S1C). The EdU staining and Annexin V-FITC/PI assay further confirmed that lnc-EBIC knockdown suppressed the tumorigenic effects of E7 on the proliferation and apoptosis of HeLa (Fig. 3B and C) and CasKi (Fig. S1D and E) cells. A series of Transwell assays were performed to evaluate the influence of lnc-EBIC on the migration and invasion of HeLa and CasKi cells. Compared with HPV16/18 E7 overexpression alone, co-transfection with lnc-EBIC siRNA significantly inhibited the migration and invasion of HeLa (Fig. 3D) and CasKi (Fig. S1F) cells. The results indicated that lnc-EBIC may be an important mediator of E7 that enhances tumorigenic activities in cervical cancer cells.

HPV16/18 E7 protein promotes lnc-EBIC expression by inhibiting TAL1 expression. HPV E7 is not a DNA-binding transcription factor (3,25). Thus, the effect of HPV E7 on lnc-EBIC expression may be mediated by cellular transcription factors. TAL1 was identified in the three databases. The present study demonstrated that E7 overexpression in HeLa (Fig. 4A-C) and CasKi cells (Fig. 4D-F) significantly decreased the expression of TAL1, with the opposite effect when E7 knockdown. To further confirm whether E7 depended on TAL1 suppression to enhance lnc-EBIC expression, lnc-EBIC levels were assessed in cervical cancer cells transfected with siRNA against TAL1

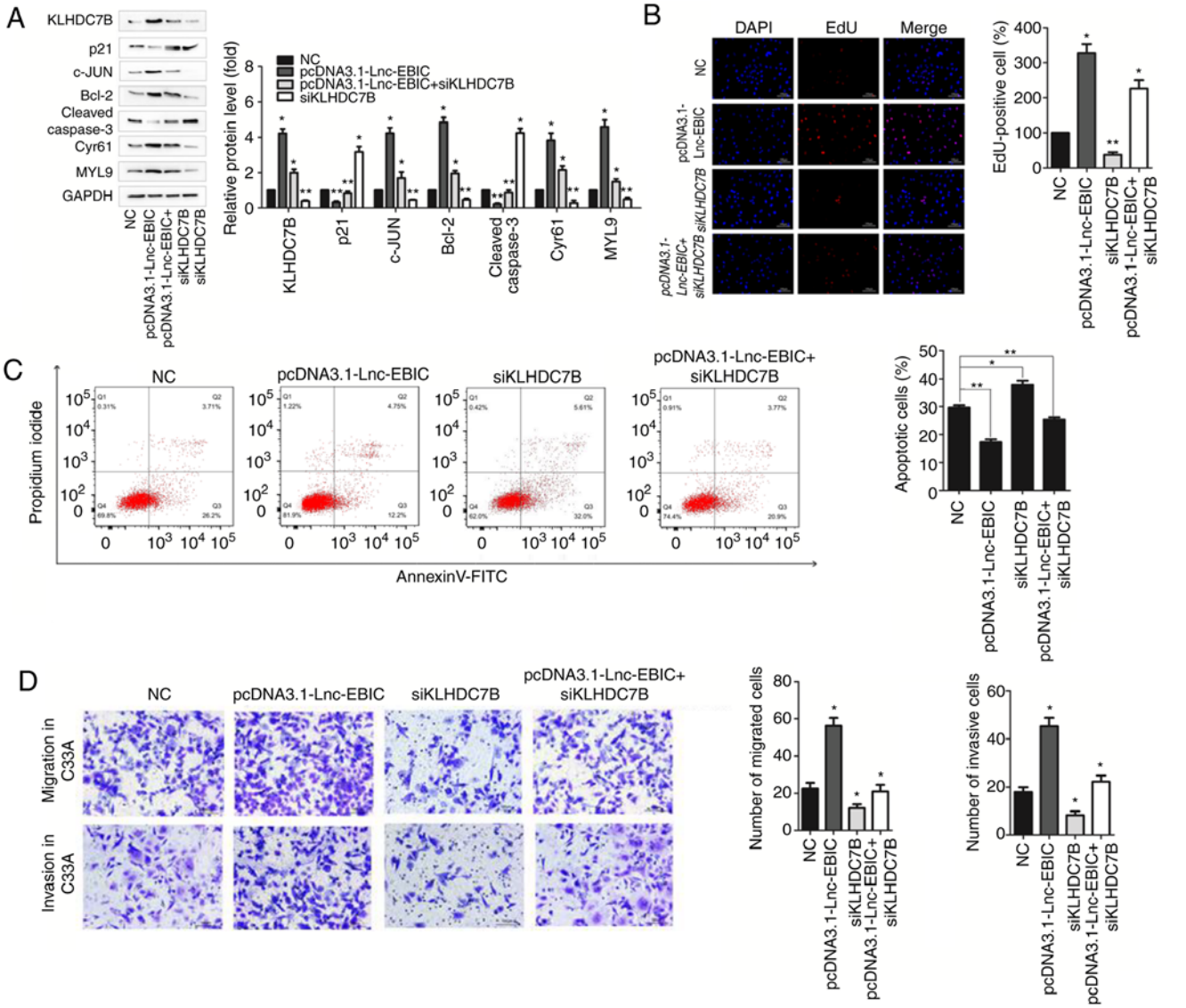

Figure 6. lnc-EBIC affects the function of cervical cancer cells by regulating KLHDC7B. (A) The western blot analysis of cellular functional proteins on cell cycle, apoptosis, migration lysates from C33A cells. (B) The fraction of S-phase lnc-EBIC-overexpressed C33A cells was increased with co-transfection of pcDNA3.1-lnc-EBIC and siKLHDC7B. (C) Interference of KLHDC7B attenuated the effect of lnc-EBIC on apoptosis in C33A cells. The percentage of cells in each quadrant is indicated. (D) Transwell assays suggested that interference of KLHDC7B attenuated the effect of lnc-EBIC on migration and invasion in C33A cells. Differences between NC, pcDNA3.1-lnc-EBIC alone and co-transfection of pcDNA3.1-lnc-EBIC and siKLHDC7B were compared. Data are presented as the means ± SD, n=3. *P<0.05 and **P<0.01. lnc, long non-coding RNA; lnc-EBIC, enhancer of zeste homolog 2-binding lncRNA in cervical cancer; KLHDC7B, Kelch domain-containing 7B; NC, negative control; si, small interfering.

alone or in the presence of E7 (Fig. 4G-I). In C33A, HeLa and CasKi cervical cancer cells, TAL1 knockdown significantly increased the expression of lnc-EBIC, an effect that was significantly suppressed following E7 inhibition (Fig. 4J-L). The results suggested that TAL1 inactivation may serve an important role in HPV E7-induced lnc-EBIC upregulation.

HPV16/18 E7 protein depends on lnc-EBIC to suppress KLHDC7B expression. Previous studies have demonstrated that KLHDC7B promotes HPV viral replication and secretion in HPV-infected cervical intraepithelial neoplasia (26). Furthermore, transcriptome sequencing analysis conducted in a previous study revealed that KLHDC7B was decreased in siRNA lnc-EBIC transfected HeLa cells (13). The expression of KLHDC7B in pcDNA3.1 and pcDNA3.1-lnc-EBIC transfected C33A cells was therefore assessed in the present study. The results revealed that the mRNA expression of KLHDC7B was significantly increased in pcDNA3.1-lnc-EBIC C33A cells compared with pcDNA3.1-transfected cells (Fig. 5A). Conversely, lnc-EBIC knockdown significantly decreased the expression of KLHDC7B in C33A cells (Fig. 5B). These results were further confirmed via western blot analysis (Fig. 5C). To determine whether the regulatory role of lnc-EBIC on KLHDC7B expression was associated with HPV16/18 E7, HeLa cells were co-transfected with Tag2B-HPV18-E7 and siRNA lnc-EBIC. Western blot analysis revealed that lnc-EBIC knockdown markedly decreased the expression of KLHDC7B in HPV18 E7-overexpression cells (Fig. 5D and E). Similar results were obtained in HPV16 E7-transfected CasKi cells (Fig. 5F and G). The results

indicated that KLHDC7B could be upregulated by lnc-EBIC and that this effect might be further enhanced by HPV16/18 E7 in cervical cancer cells.

KLHDC7B cooperates with lnc-EBIC to promote the tumorigenic activities of cervical cancer cells. To elucidate whether KLHDC7B was involved in lnc-EBIC-mediated tumorigenic activity, C33A cells were transfected with the lnc-EBIC overexpression plasmid alone or in the presence of siRNA KLHDC7B. As presented in Fig. 6A, lnc-EBIC overexpression significantly increased the expression of anti-apoptotic c-JUN, Bcl-2, Cyr61 and MYL9, and reduced the expression of pro-apoptotic p21 and Cleaved caspase-3 in C33A cells. Moreover, as predicted, KLHDC7B knockdown significantly suppressed the effects of lnc-EBIC on the expression of apoptotic proteins (Fig. 6A) and markedly inhibited the tumor-promotive effects of lnc-EBIC, including increased cellular proliferation (Fig. 6B), migration and invasion (Fig. 6D), and decreased apoptosis (Fig. 6C) in C33A cells. The results demonstrated that KLHDC7B served an important role in the lnc-EBIC-mediated tumorigenic activities of cervical cancer cells.

Discussion

lncRNAs serve key regulatory roles in the occurrence and progression of cervical cancer. For example, breast cancer anti-estrogen resistance 4, a lapatinib-responsive lncRNA (an EGFR/HER2 inhibitor) enhanced cell proliferation in estrogen-resistant breast cancer, and serves as a metastasis-promoting lncRNA in cervical cancer (27). A nine-lncRNA signature composed of ATXN8 opposite strand lncRNA, chromosome 5 Open Reading Frame 60, DIO3 opposite strand upstream RNA, EMX2 opposite strand/antisense RNA, inactivation escape 1, KCNQ1 downstream neighbor protein, KCNQ1 overlapping transcript 1, loss of heterozygosity on chromosome 12 region 2 and RFPL1 antisense RNA 1 exhibits great potency for the prediction of cervical cancer recurrence (28). Moreover, recent studies have indicated that lncRNAs including growth arrest-specific 5, H19 imprinted maternally expressed transcript (non-protein coding), FAM83H antisense RNA 1, metastasis-associated lung adenocarcinoma transcript 1 and CCEPR (11,12) can be exploited by HPVs to perform tumorigenic activities. However, these lncRNAs are specifically regulated by E6; less is known about the lncRNAs that are regulated by E7. The present study revealed that oncogenic lnc-EBIC could be exploited by HPV16/18 E7 to accelerate the proliferation, migration and invasion, and inhibit apoptosis in cervical cancer cells. lnc-EBIC also exhibited oncogenic activities even in HPV^- cervical cancer cells. Therefore, lnc-EBIC may be a novel therapeutic target for patients with HPV^+ and HPV^- cervical cancers.

lncRNAs regulate a variety of critical cellular processes by promoting or repressing transcription, serving as epigenetic regulators or as scaffolds to interact with various proteins in cervical cancer (29,30). Lnc-cervical cancer DExH-box helicase 9 (DHX9) suppressive transcript (lnc-CCDST), a recently identified tumor-suppressive lncRNA that can be abolished by E7, has been revealed to promote pro-oncogenic DHX9 degradation by serving as a scaffold to facilitate the formation of mouse double minute 2 (MDM2) and DHX9 complexes, while not influencing the mRNA expression of DHX9 (31). Lnc-EBIC has been confirmed to act as a competing endogenous RNA that sequesters tumor repressors miR-375 and miR-139, which target HPV16/18 E6/E7 mRNA (13), and to serve as a scaffold that interacts with EZH2, thus repressing E-cadherin expression (14,15). The present study further demonstrated that lnc-EBIC promoted cellular proliferation, migration and invasion, and inhibited apoptosis in cervical cancer cells by enhancing KLHDC7B expression.

The transcription factor TAL1 is an essential regulator of hematopoiesis that promotes prostate cancer cell growth via the MAPK/ERK, PI3K/AKT and AMPK signaling pathways (32). However, its role in cervical cancer remains unknown. The present study revealed that TAL1 was significantly downregulated in HPV E7 cervical cells. Furthermore, it was demonstrated that the inactivation of TAL1 may serve an important role in HPV E7-induced lnc-EBIC upregulation.

KLHDC7B is associated with an aggressive subtype of cancer and predicts a poor prognosis in patients with breast (16) and laryngeal (33) cancers. Furthermore, KLHDC7B is upregulated in HPV-induced vulvar intraepithelial neoplasia (26) and can be used as a biomarker for the diagnosis and prognostic prediction of patients with cervical cancer (19). In the present study, interfering KLHDC7B expression was observed to significantly inhibit the oncogenic activities of lnc-EBIC. Thus, KLHDC7B may be a pivotal target of lnc-EBIC in cervical cancer cells. As both mRNA and protein levels of KLHDC7B were enhanced by lnc-EBIC, the exact interaction pathway between lnc-EBIC and KLHDC7B requires further elucidation.

E7 performs oncogenic activities by modulating the expression of several host proteins, including pRB, p107, p130, p21, octamer-binding transcription factor 4 and PTPN14 (34,35). The present study demonstrated that HPV E7 was dependent on the inhibition of TAL1 to promote lnc-EBIC expression. TAL1 is a transcription factor that is aberrantly expressed in 60% of cases of human T-cell acute lymphoblastic leukemia (T-ALL) cases, activating several important oncogenes, including the MYC, MYB, Notch1, cyclin E, and tribbles pseudokinase 2 (36,37). Bioinformatic analysis was performed in the present study to identify the lncRNAs that are regulated by TAL1 in T-ALL cells (38). The results revealed that lnc-EBIC was one such lncRNA. Additionally, a transcriptome profiling study determined that TAL1 was overexpressed in gastric-type cervical cancer that was not associated with HPV infection (39). TAL1 knockdown in HPV^+ (HeLa and CasKi) and HPV^- (C33A) cells in the present study induced a significant increase in lnc-EBIC expression. TAL1 may therefore represent a novel target for E7 in HPV infection. However, its role in cervical cancer progression requires further clarification.

In conclusion, the present study revealed that oncogenic lnc-EBIC can be exploited by HPV16/18 E7 to increase cellular proliferation, migration and invasion, and decrease apoptosis in cervical cancer cells. Molecular analysis revealed that E7 is dependent on the TAL1/lnc-EBIC/KLHDC7B axis to perform its tumor-promotive activities. Furthermore, lnc-EBIC exhibited oncogenic activity by enhancing KLHDC7B expression in HPV^- cervical cancer cells. Thus, the lnc-EBIC/KLHDC7B axis represents a novel molecular mechanism and potential therapeutic target for both HPV^+ and HPV^- cervical cancer.

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Availability of data and materials

All the datasets generated and/or analyzed during the present study are included in this published article.

Authors' contributions

JW, FX and XL performed the experiments, contributed to data analysis and wrote the manuscript. XM, XC, XS and YY analyzed the data. CY, YX and HX conceptualized the study design, contributed to data analysis and experimental materials. All authors have read and approved the final version of this manuscript.

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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