ElncRNA1, a long non-coding RNA that is transcriptionally induced by oestrogen, promotes epithelial ovarian cancer cell proliferation

JUN-JUN QIU[1-3], XIAO-DAN ZHANG[1-3], XIAO-YAN TANG[1-3],
TING-TING ZHENG[1-3], YING ZHANG[1-3] and KE-QIN HUA[1-3]

[1]Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, Shanghai 200011;
[2]Department of Obstetrics and Gynecology of Shanghai Medical College, Fudan University, Shanghai 200032;
[3]Shanghai Key Laboratory of Female Reproductive Endocrine-Related Diseases, Shanghai 200011, P.R. China

Abstract. Previously, the novel oestrogen (E2)-upregulated lncRNA TC0101441, was identified by us, via microarray analysis. However, the detailed mechanism by which E2 upregulates TC0101441 and the role of TC0101441 in epithelial ovarian cancer (EOC) progression have not been elucidated. In the present study, we further analysed TC0101441, which we designated oestrogen-induced long non-coding RNA-1 (ElncRNA1). We showed that E2 transcriptionally upregulates ElncRNA1 through the oestrogen receptor α (ERα)-oestrogen response element (ERE) pathway using RNA stability assays, bioinformatics-based searches for ERE binding sites, chromatin immunoprecipitation (ChIP) assays and dual luciferase reporter assays. Clinically, ElncRNA1 levels are significantly higher in EOC tissues than in normal ovarian surface epithelium. *In vitro* and *in vivo* loss-of-function assays revealed that ElncRNA1 promotes EOC cell proliferation. This pro-proliferation effect of ElncRNA1 was partially mediated by the regulation of CDK4, CDK6 and cyclin D1. These findings provide the first evidence that E2 upregulates ElncRNA1 at the transcriptional level through the ERα-ERE pathway and that this novel E2-upregulated lncRNA has an oncogenic role in EOC growth. The placement of ElncRNA1 in the E2-ERα-ERE signalling pathway may provide greater insight into the effects of oestrogen on EOC progression from the perspective of lncRNA.

Introduction

Epithelial ovarian cancer (EOC) is the leading cause of death from gynaecologic malignancies (1-3). EOC is highly lethal, primarily because it is diagnosed at an advanced stage and is characterized by aggressive proliferation and metastasis (2). Despite advances in therapy, the high mortality and poor prognosis associated with EOC remain unchanged (2). Therefore, identifying new therapeutic targets for EOC and understanding the molecular mechanisms underlying EOC aggressiveness will yield novel strategies for overcoming this malignancy.

Accumulating clinical and epidemiological evidence has shown that oestrogen (E2) is responsible for promoting ovarian cancer progression (4,5). *In vitro* and *in vivo* experimental data have indicated that E2 enhances EOC cell proliferation (6-11). The effects of E2 on EOC proliferation are mainly attributable to E2-regulated target genes. Previous studies have reported numerous E2-regulated proteins that affect EOC cell proliferation and progression, such as cyclin D1, c-myc and insulin-like growth factor-binding protein (IGFBP) family members (5,12). Despite the identification of these E2-regulated proteins, the exact effect of E2 on EOC progression is not fully understood. Therefore, there is an urgent need to identify novel E2-regulated target genes.

The discovery of long non-coding RNAs (lncRNAs, >200 nucleotides) has opened a new avenue in cancer research. Accumulating evidence highlights the importance of lncRNAs as a novel class of oncogenes or tumour suppressor genes in the development of many types of cancer (13-23), including EOC (24-28). Further, some lncRNAs have recently been reported to be involved in E2 signalling. For example, the lncRNA-SRA1 was reported to function as a nuclear receptor corepressor in E2 signalling (29), and HOTAIR is upregulated by E2 and contributes to E2-supported breast cancer progression (30,31). However, little is known about E2-regulated lncRNAs in ovarian cancer.

We previously identified a novel E2-upregulated lncRNA, TC0101441, based on microarray analysis and found that TC0101441 contributes to E2-induced EOC cell migration (32). However, the detailed mechanism by which E2 upregulates TC0101441 and the role of ElncRNA1 itself in EOC progression have not been elucidated. In the present study, we further evaluated TC0101441, designated as oestrogen-induced long non-coding RNA-1 (ElncRNA1), and showed that E2

Correspondence to: Dr Ke-qin Hua, Department of Gynecology, Obstetrics and Gynecology Hospital of Fudan University, 419 Fangxie Road, Shanghai 200011, P.R. China
E-mail: huakeqin@126.com

upregulated ElncRNA1 at the transcriptional level through the oestrogen receptor α (ERα)-oestrogen response element (ERE) pathway. Clinically, ElncRNA1 levels were significantly higher in EOC tissues than in normal ovarian surface epithelial tissues. *In vitro* and *in vivo* assays revealed that ElncRNA1 promotes EOC cell proliferation. This pro-proliferation effect of ElncRNA1 was partially mediated by the regulation of CDK4, CDK6 and cyclin D1. These findings not only clarify the mechanism by which E2 upregulates ElncRNA1 but also underscore the important role of this novel E2-upregulated lncRNA in EOC proliferation, thus providing a connection between E2 and ovarian cancer from the perspective of lncRNA.

Materials and methods

Cell lines and clinical tissue samples. The human EOC cell lines (SKOV3, CAOV3, OVCAR3 and HO8910) were a gift from University of Texas MD Anderson Cancer Center (Houston, TX, USA). SKOV3, CAOV3 and HO8910 cells were cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA). OVCAR3 cells were maintained in McCoy's 5A supplemented with 10% foetal bovine serum (Gibco) at 37˚C in a 5% CO_2 atmosphere. For E2 treatment, cells (plated at 20-30% confluence) were grown for 3 days in phenol red-free RPMI-1640 or McCoy's 5A containing 5% charcoal-stripped foetal bovine serum (Serana, Bunbury, Australia). Next, the cells were treated for 24 h with 10^{-8} M E2 (Sigma) or vehicle (DMSO, 0.01% of final volume) as a control.

All tissue samples were obtained during surgical operations at the Obstetrics and Gynecology Hospital of Fudan University between August 2005 and December 2008. All EOC tissues were selected from patients who a) had not received preoperative radiotherapy, chemotherapy, or hormonal therapy; and b) did not have borderline ovarian tumours or two or more different malignancies. All 40 EOC tissues analysed herein were serous ovarian cancer tissues. In particular, according to the International Federation of Gynecologists and Obstetricians (FIGO) staging system, 6 cases were FIGO stage I, 6 cases were FIGO stage II, 24 cases were FIGO stage III, and 4 cases were FIGO stage IV. Additionally, according to histological grading guidelines, 6 cases were grade I (G1), 12 cases were G2, and 22 cases were G3. The normal ovarian epithelial tissues were obtained from participants diagnosed with uterine fibroids who were scheduled to undergo a hysterectomy and oophorectomy. Tissues were selected from participants who did not have a history of ovarian cysts, ovarian pathology, or ovarian surgery. All samples were pathologically confirmed, immediately frozen in liquid nitrogen and stored at -80˚C. The study was approved by the Research Ethics Committee of Fudan University, China. Informed consent was obtained from each patient.

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and then reverse transcribed into cDNA using the ExScript RT-PCR kit (Takara, Otsu, Japan) following the manufacturer's instructions. ElncRNA1 expression was measured by qRT-PCR using the following primer sequences: forward, 5'-CAAGGCAGGTGA GAACGAGT-3'; and reverse, 5'-CTCGACTTAGGGAGCT

GCAC-3'. Expression of the internal control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), was measured with the following primers: forward, 5'-TGACTTCAACAGCGA CACCCA-3'; and reverse, 5'-CACCCTGTTGCTGTAGCC AAA-3'. Amplification and detection were conducted using a Prism 7900 system (Applied Biosystems, Foster City, CA, USA) with the ExScript SYBR Green qRT-PCR kit (Takara). All assays were repeated in triplicate, and statistical analyses of the results were performed using the $2^{-\Delta\Delta Ct}$ relative quantification method.

Sequence analysis of ElncRNA1. The gene location was obtained from the University of California Santa Cruz (UCSC) genome browser (hg18, http://genome.ucsc.edu/). The Basic Local Alignment Search Tool (BLAST) was used for sequence alignment, and the BLAST-Like Alignment Tool (BLAT) was used to map the cDNA to chromosomes.

RNA stability assay. To measure RNA half-life, we added 10 μg/ml actinomycin D (Sigma) to the cells to block transcription (nascent RNA synthesis). The cells were pre-treated with actinomycin D prior to E2 treatment. Total RNA was extracted from samples harvested at the indicated time points. ElncRNA1 expression was quantified by qRT-PCR. The half-life of ElncRNA1 RNA was calculated using data from two independent experiments.

Search for EREs. A region 2,500 bp upstream and 500 bp downstream of the transcriptional start site (TSS) was screened for transcription factor binding sites (TFBSs) using the Patser program (http://ural.wustl.edu/src/patser-v3e.1.tar. gz) and TRANSFAC 8.1 position weight matrices (PWMs) (http://www.gene-regulation.com/). A cut-off score of 0.9 was employed, and both the forward and reverse genomic DNA strands were investigated. EMBOSS/fuzznuc software (http:// helixweb.nih.gov/emboss/html/fuzznuc.html) was used to evaluate the putative EREs in the E2-regulated lncRNA. The conserved ERE sequence is AGGTCAnnnTGACCT, and up to two mismatches were allowed.

Plasmids and luciferase assays. A firefly luciferase (Luc) reporter driven by a fragment of the 3' untranslated regions (UTR) of ElncRNA1 containing the putative ERE [Luc-ElncRNA1-3'UTR wild-type (WT)] and a Luc reporter driven by a corresponding mutant sequence (Luc-ElncRNA1- 3'UTR Mut) were synthesized. The primer sequences were as follows: sense for Luc-ElncRNA1-3'UTR WT and Luc-ElncRNA1-3'UTR Mut: GGGGTACCGTAGAGACAGG GTCTCACCACATT; antisense for Luc-ElncRNA1-3'UTR WT: CGACGCGTGGAGCATCTGTCCCAGCTG; and antisense for Luc-ElncRNA1-3'UTR Mut: CGACGCGTGGAGC ATCTGTCCCAGCTGGAGTCCCCTGTGGTGTTATCTG AGATTCAGCAGCTCTGCAAGAATATCATCTATGAC. A plasmid expressing ERα was constructed by Hanyin Co. (Shanghai, China). A plasmid expressing Renilla Luc was purchased from Promega (Madison, WI, USA). SKOV3 cells were transfected with the above plasmids or vectors for 24 h and then treated with E2; Luc assays were performed after a 16 h E2 treatment using a Dual-Luciferase Assay kit (Promega) according to the manufacturer's protocol.
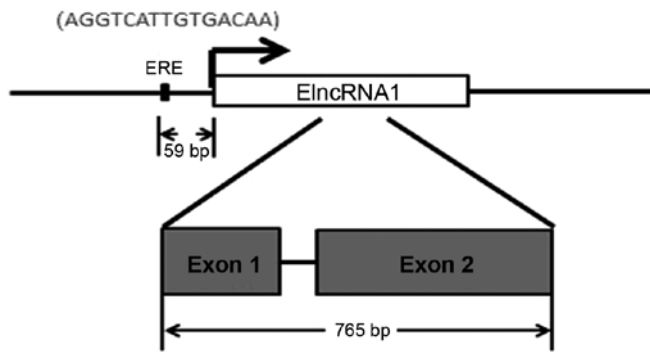
Figure 1. Characterization of the ElncRNA1 transcript. Diagram of the ElncRNA1 gene locus. The ElncRNA1 gene consists of 2 exons. The predicted ERE is located in the 59 bp region upstream of the TSS of ElncRNA1.
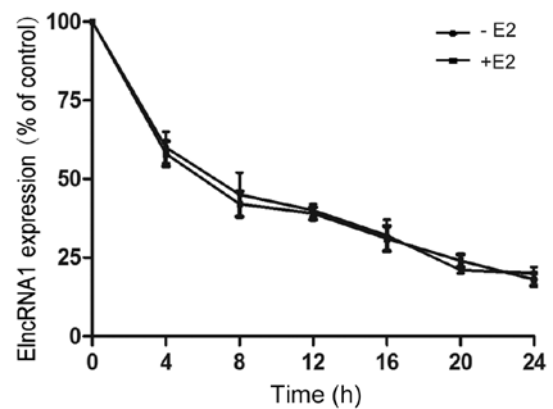

Figure 2. Effects of oestrogen on ElncRNA1 mRNA stability. The Y-axis shows relative ElncRNA1 expression levels in SKOV3 cells after incubation with actinomycin D for various times.

Chromatin immunoprecipitation (ChIP). ChIP assays were performed using a ChIP assay kit (Millipore) according to the manufacturer's instruction. Specific antibodies were used to immunoprecipitate either ERα or the negative control IgG. Real-time PCR was performed using a SYBR Green PCR kit (Qiagen). The primer sequences for amplifying the ElncRNA1 promoter region flanking the ERα-ERE binding sites were as follows: forward, 5'-GGAAGAACAGCTCCGTGAAG-3'; and reverse, 5'-CAGATTCAGGGCTCTTGAGG-3'.

Establishment of stable ElncRNA1-knockdown cell lines. The two ElncRNA1-siRNA sequences were 5'-GCTCACATGAGAAAGCAAACT-3' (siRNA1) and 5'-CUUGAGUUAUGAGGUAGCA-3' (siRNA2). Lentiviral vectors encoding ElncRNA1-shRNA were designed based on the two siRNA sequences (ElncRNA1-knockdown (KD)1 and ElncRNA1-KD2) and were constructed by Hanyin Co. The recombinant lentiviruses (KD1 and KD2) and the negative control (NC) lentivirus (Hanyin Co.) were prepared at a titre of 10^9 transfection units (TU)/ml. To obtain stable cell lines, SKOV3 and CAOV3 cells were seeded in 6-well plates and infected with virus and polybrene the following day. Positive clones were selected with puromycin for 14 days to establish the following new stable cell lines: SKOV3-NC, SKOV3-KD1, SKOV3-KD2, CAOV3-NC, CAOV3-KD1 and CAOV3-KD2.

MTT assay. Cell proliferation was assayed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich, St. Louis, MO, USA) and a Synergy H4 Hybrid Reader. Briefly, the culture medium was removed after 1, 2, 3, 4, and 5 days; 0.5 mg/ml MTT in 200 μl of medium was added to each well; and the plates were incubated for 4 h. Then, 150 μl of DMSO was added to the SKOV3 and CAOV3 cells for 10 min, and the optical density (OD) was measured at 490 nm. Each experiment was repeated in triplicate.

Colony formation assay. Duplicate cultures of each cell type were maintained at 37°C in a 5% CO_2 atmosphere, and fresh medium was added every 3 days. After 2 weeks, colonies in each well consisting of >50 cells were counted. Each experiment was repeated in triplicate.

Tumour formation in nude mice. The animal experiments were approved by the Institutional Animal Care and Use Committee of Fudan University and were performed according to the institutional guidelines and protocols. SKOV3-KD1, SKOV3-KD2, and SKOV3-NC cells ($1x10^6$) were subcutaneously injected into 5-week-old BALB/c athymic nude mice (Department of Laboratory Animals, Fudan University; n=7 for each cell line). The mice were sacrificed after 4 weeks and examined for the growth of subcutaneous tumours. The tumour volume was calculated as previously described (33).

Western blotting. Western blot assays were performed using the following primary antibodies: anti-cyclin E (Santa Cruz); anti-cyclin D1 (Cell Signaling Technology); anti-CDK2 (Abways Technology); anti-CDK4 (Abways Technology); anti-CDK6 (Abways Technology); and β-actin (Proteintech). Briefly, stimulated cells were lysed with RIPA buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate] containing protease inhibitors (Roche, Complete Mini); 20-30 μg samples of the lysates were separated on 8-12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were incubated with primary antibodies overnight at 4°C. The primary antibody incubation was followed by incubation with an HRP-conjugated secondary antibody. The bound antibodies were detected using an ECL kit (Pierce).

Statistical analysis. The data were processed using SPSS version 16.0 software (SPSS, Inc., Chicago, IL, USA). Continuous data were compared between the two groups using independent t-tests. P-values at <0.05 were considered statistically significant.

Results

A novel E2-induced lncRNA, TC0101441/ElncRNA1. We previously identified a novel lncRNA, TC0101441, which was significantly upregulated by E2 in SKOV3 ERα-positive ovarian cancer cells (32). In this study, we designated TC0101441 as E2-induced lncRNA 1 (ElncRNA1). Based on a bioinformatics analysis using the UCSC genome browser (hg18, http://genome.ucsc.edu/), we noted that ElncRNA1 is located on chromosome 1 (chr1: 202,377,159:202,378,011), contains two exons and encodes a 765 bp lncRNA molecule (Fig. 1).
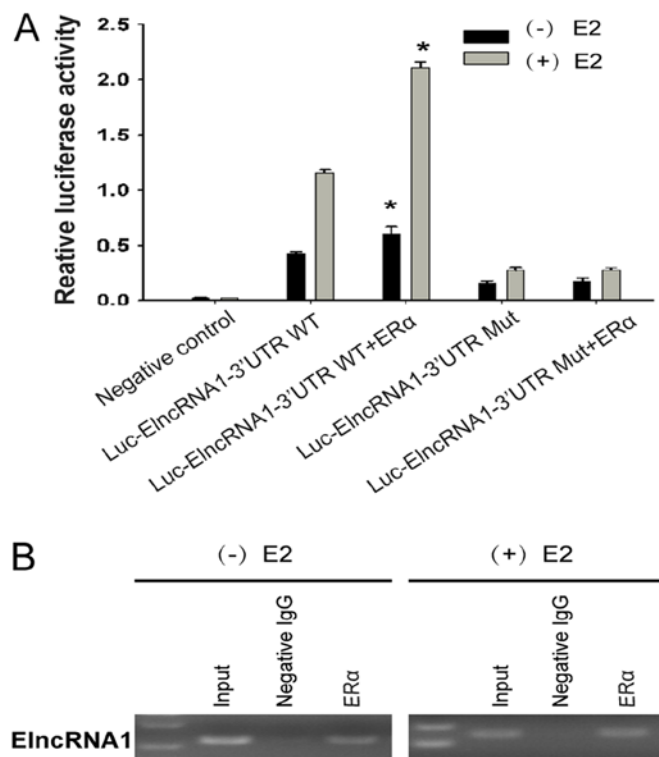
Figure 4. Relative ElncRNA1 expression in ovarian cancer tissues. ElncRNA1 expression in ovarian cancer tissues and normal ovarian tissues was determined by qRT-PCR.

Figure 3. ERα-ERE is required for the transcriptional regulation of ElncRNA1 by E2. (A) SKOV3 cells were transfected with ERα and ElncRNA1 promoter reporters containing a mutated or wild-type ERE. Luciferase activity was measured and normalized to Renilla luciferase activity. *P<0.05. (B) ChIP analysis of ERα binding to the ElncRNA1 promoter. ChIP assays were performed with antibodies against ERα to confirm ERα binding to the ERE region within the ElncRNA1 promoter. Enrichment of ERα-associated promoter fragments was confirmed by RT-PCR.

Effects of E2 on ElncRNA1 mRNA stability. To determine whether E2-mediated changes in ElncRNA1 levels are related to transcription or mRNA stability, we treated SKOV3 cells with actinomycin D to block nascent RNA synthesis prior to E2 treatment and measured ElncRNA1 levels at various subsequent time points. RNA stability was not significantly altered in SKOV3 cells in the absence or presence of E2 (Fig. 2). This result suggests that the E2-mediated changes in ElncRNA1 levels are not due to altered mRNA stability and implies that transcriptional regulation may be a major mechanism by which ElncRNA1 is induced.

ERα-ERE binding is required for transcriptional regulation of ElncRNA1 expression by E2. Given the results of our prior study, which demonstrated that E2 induced ElncRNA1 expression in an ERα-dependent manner (32), we next screened the ElncRNA1 promoter for the presence of ERα-responsive elements to confirm the transcriptional upregulation of ElncRNA1 by E2. We identified a predicted ERE (-1725 to -1711) in a region 59 bp upstream of the TSS of ElncRNA1 (Fig. 1). We subsequently transfected SKOV3 cells with ERα and ElncRNA1 promoter reporters containing a mutated or wild-type (WT) ERE and then measured Luc activity after E2 treatment. Cells expressing both WT ERE and ERα responded strongly to E2 by inducing reporter activity (Fig. 3A), suggesting that both ERα and ERE are required for E2 to upregulate ElncRNA1 expression.
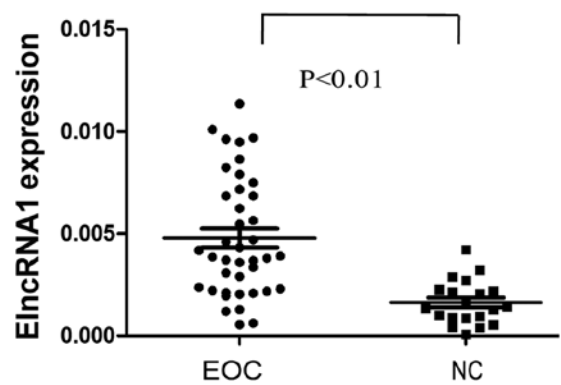
We subsequently performed ChIP assays with antibodies against ERα to confirm that ERα binds to the ERE region of the ElncRNA1 promoter. As shown in Fig. 3B, enrichment of ERα-associated promoter fragments was confirmed by RT-PCR, and this enrichment was enhanced by E2. These results indicate that ERα can bind to the ERE in the ElncRNA1 promoter in a manner that is enhanced by E2 treatment, thus inducing ElncRNA1 expression.

ElncRNA1 expression pattern in EOC tissues. To investigate whether ElncRNA1 has clinical implications, we used qRT-PCR to determine the ElncRNA1 expression pattern in EOC. ElncRNA1 levels were significantly higher in 40 EOC tissues than in 20 normal ovarian surface epithelial tissues (P<0.01; Fig. 4). These results suggest that ElncRNA1 overexpression may play a role in EOC aggressiveness.

Silencing ElncRNA1 suppresses EOC cell proliferation in vitro. To determine whether ElncRNA1 affects EOC cell proliferation, we first examined the basal levels of ElncRNA1 in 4 EOC cell lines (SKOV3, CAOV3, OVCAR3 and HO8910) and found that SKOV3 and CAOV3 cells expressed relatively higher ElncRNA1 levels (Fig. 5A). Thus, we silenced ElncRNA1 expression in these two cell lines to investigate the effects of ElncRNA1 on cell proliferation. Since both siRNAs efficiently silenced ElncRNA1 expression in SKOV3 and CAOV3 cells (Fig. 5B), we constructed lentiviral vectors to establish stable ElncRNA1-knockdown cell lines (SKOV3-KD1, SKOV3-KD2, CAOV3-KD1, and CAOV3-KD2 cells) and corresponding controls (SKOV3-NC and CAOV3-NC cells).

MTT assays showed that ElncRNA1 knockdown significantly suppressed the proliferation of both SKOV3 and CAOV3 cells (Fig. 5C). Colony formation assays demonstrated that ElncRNA1 knockdown reduced the number of SKOV3 and CAOV3 colonies (Fig. 5D). These results indicate that silencing ElncRNA1 may inhibit EOC cell proliferation *in vitro*.

Silencing ElncRNA1 inhibits EOC tumour growth in vivo. Next, we tested the effects of ElncRNA1 on EOC tumour growth *in vivo* by injecting SKOV3-KD1, SKOV3-KD2 and SKOV3-NC cells into nude mice. ElncRNA1 knockdown reduced the frequency of tumour formation (SKOV3-NC, 7/7;
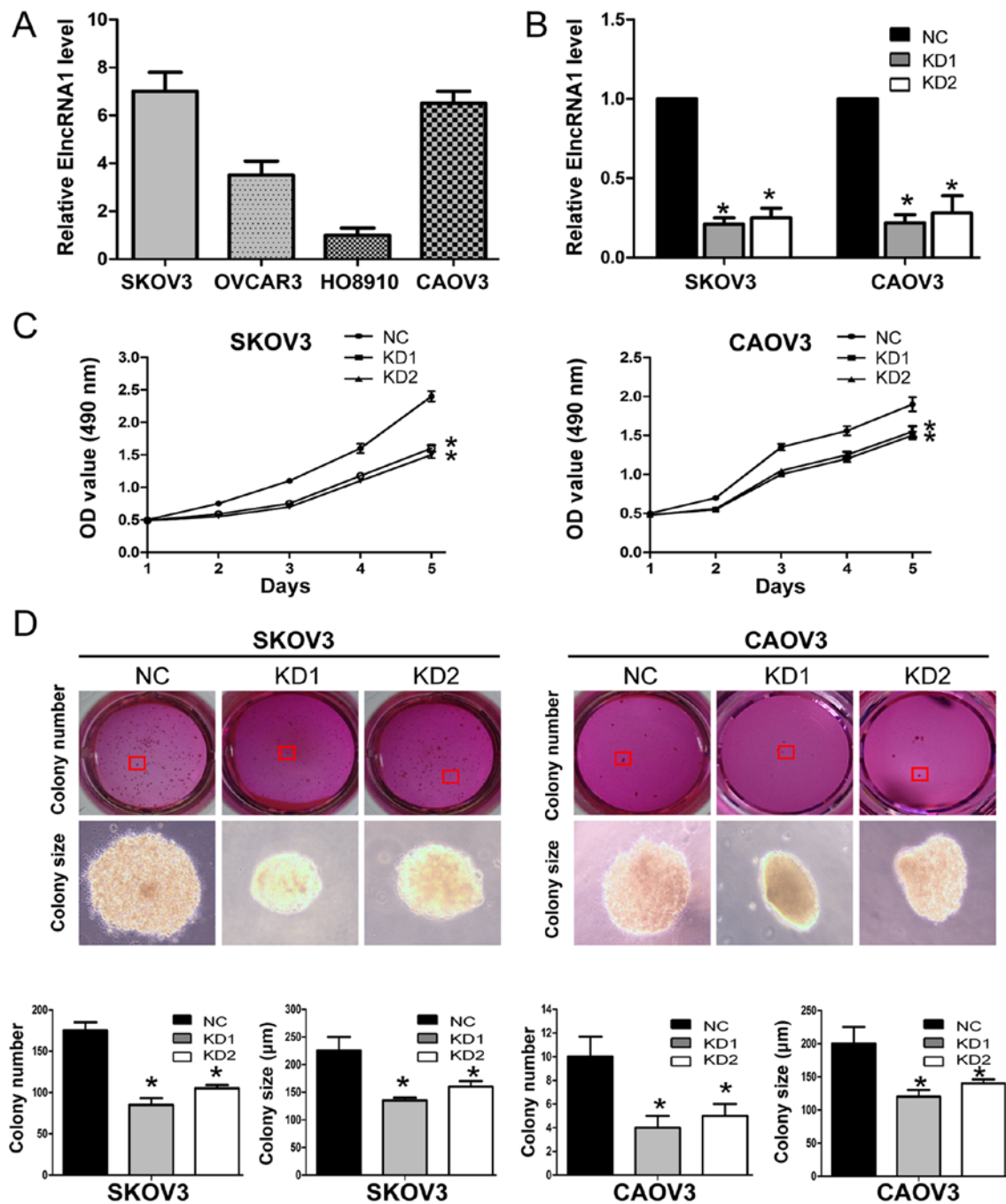
Figure 5. The effect of ElncRNA1 on ovarian cancer cell proliferation. (A) Relative ElncRNA1 expression levels in epithelial ovarian cancer cell lines (SKOV3, CAOV3, OVCAR3 and HO8910). (B) Relative ElncRNA1 expression levels in SKOV3 and CAOV3 cells transfected with si-NC or ElncRNA1-siRNAs. *P<0.05. (C) MTT assays were performed to evaluate the proliferation of SKOV3-KD and CAOV3-KD cells compared with control cells. The data are presented as the mean ± standard deviation (SD) of three independent experiments. The bars denote the SD. *P<0.05. (D) Representative colony formation assay results, including colony number and size, for SKOV3-KD and CAOV3-KD cells and their corresponding controls. *P<0.05.

SKOV3-KD1, 4/7; and SKOV3-KD2, 6/7) (Fig. 6A). Moreover, qRT-PCR analysis confirmed that ElncRNA1 expression levels were significantly lower in tumour tissues from the ElncRNA1-KD group relative to those from the control group (Fig. 6B). Further, the volume and average weight of tumours formed by SKOV3-KD1 and SKOV3-KD2 cells were much lower compared to tumours formed by SKOV3-NC cells (Fig. 6C). Together, these results suggest that silencing ElncRNA1 inhibits EOC growth *in vivo*, which corresponds with the *in vitro* results.

Certain proliferation-related proteins are downstream mediators of ElncRNA1 activity affecting EOC cell proliferation. To study possible mechanisms through which ElncRNA1 alters EOC cell proliferation further, we evaluated the expression of certain key proliferation-related proteins, including cyclin D1, cyclin E, CDK2, CDK4, CDK6, by western blot assay. The results showed that ElncRNA1 knockdown in SKOV3 and CAOV3 cells resulted in a significant decrease in CDK4, CDK6 and cyclin D1 protein levels (Fig. 7), suggesting that CDK4, CDK6 and cyclin D1 are the downstream media-
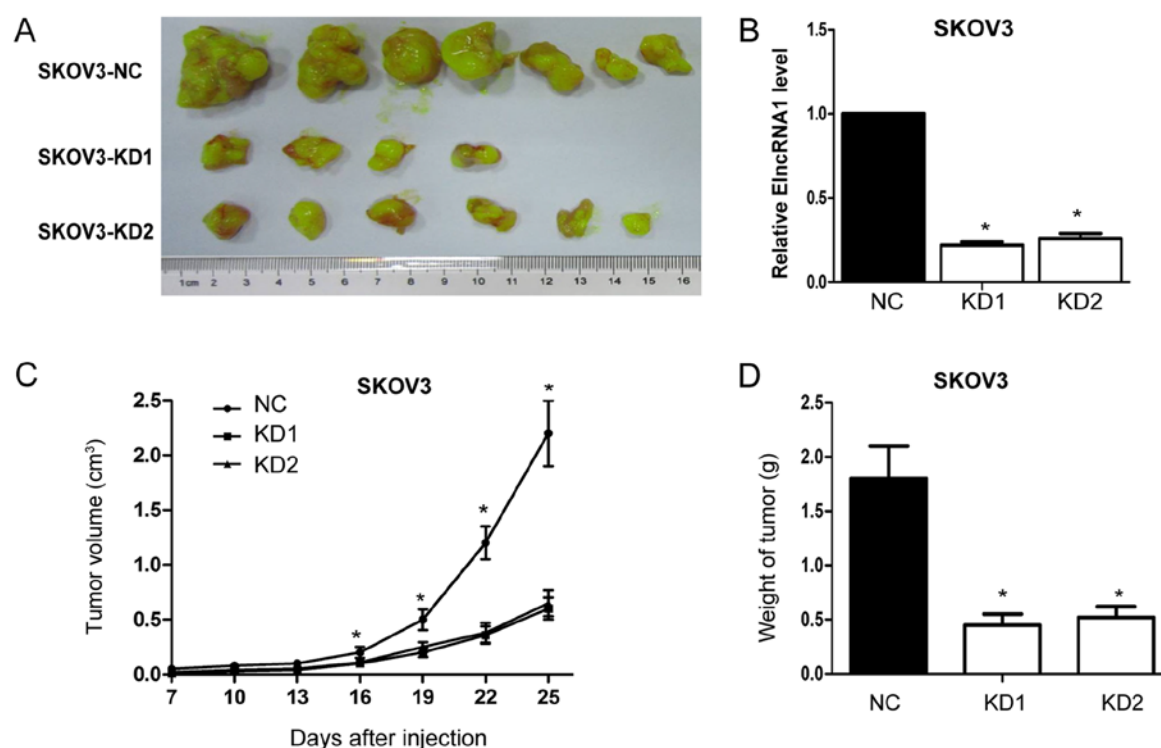
Figure 6. ElncRNA1 knockdown inhibits SKOV3 cell proliferation *in vivo*. (A) The frequency of tumour formation in the SKOV3-NC, SKOV3-KD1 and SKOV3-KD2 groups. (B) ElncRNA1 expression levels were lower in SKOV3-KD1 and SKOV3-KD2 tumours than in SKOV3-NC tumours. *P<0.05. (C) Growth curves of tumour xenografts. The volume of SKOV3-KD1 and SKOV3-KD2 tumours was substantially less than that of SKOV3-NC tumours, and the gap in tumour size between the KD groups and the NC group increased over time (*P<0.05). (D) SKOV3-NC tumours were significantly heavier than SKOV3-KD1 and SKOV3-KD2 tumours. *P<0.05.

tors of ElncRNA1 activity affecting EOC cell proliferation. Together, these data indicate that ElncRNA1 regulates EOC cell proliferation at least in part by regulating CDK4, CDK6 and cyclin D1.

Discussion

Previously, we identified the novel E2-upregulated lncRNA, ElncRNA1, based on microarray analysis. However, the detailed mechanisms by which E2 upregulates ElncRNA1 and the role of ElncRNA1 itself in EOC progression have not been determined (32). In the present study, using RNA stability assays, bioinformatics-based searches for ERE binding sites, ChIP assays and dual luciferase reporter assays, we found that E2 transcriptionally upregulated ElncRNA1 through the ERα-ERE pathway. Clinically, ElncRNA1 levels were significantly higher in EOC tissues than in normal ovarian surface epithelial tissues. Further *in vitro* and *in vivo* assays revealed that ElncRNA1 promoted EOC cell proliferation. This pro-proliferation effect of ElncRNA1 was partially mediated by the regulation of CDK4, CDK6 and cyclin D1. Together, these findings not only clarify the mechanism by which E2 upregulates ElncRNA1 but also underscore the important role of this novel E2-upregulated lncRNA in EOC proliferation, thus providing a connection between E2 and ovarian cancer from the perspective of lncRNA.

E2 plays an important role in regulating cancer growth through various target genes, including protein coding genes and non-coding RNAs. We previously identified a novel E2-upregulated lncRNA (TC0101441, designated
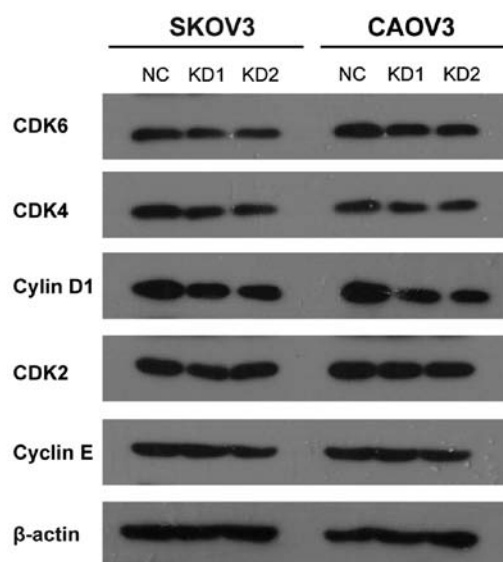

Figure 7. ElncRNA1 knockdown alters CDK4, CDK6 and cyclin D1 expression. Western blots showing that ElncRNA1 knockdown decreases CDK4, CDK6 and cyclin D1 expression levels in SKOV3 and CAOV3 cells.

ElncRNA1) (32), but the molecular mechanisms by which E2 upregulates ElncRNA1 have not been determined. So far, several mechanisms have been proposed for E2-regulated gene expression, including direct action of basal transcriptional elements and the participation of cofactors (34-36). Our prior study demonstrated that the E2-mediated induction of ElncRNA1 expression is dependent on ERα (32), a

ligand-activated transcription factor in the nuclear receptor super-family that directly binds to oestrogen-responsive elements (EREs) in the promoter regions of target genes, thereby regulating their transcription (34,36,37). Therefore, we hypothesized that ERα-ERE binding might contribute to the transcriptional upregulation of ElncRNA1 expression by E2. To address this hypothesis, we initially performed mRNA stability experiments. The use of actinomycin D suggested that the E2-induced changes in ElncRNA1 expression were not due to effects on mRNA stability but were rather due to changes in E2-regulated transcription. To confirm the transcriptional upregulation of ElncRNA1 by E2, we screened the ElncRNA1 promoter and found an ERE in a 59 bp region upstream of the TSS of ElncRNA1. Subsequent ChIP assays indicated that ERα bound to the ERE in the ElncRNA1 promoter. Moreover, dual luciferase reporter assays revealed that cells expressing both WT ERE and ERα responded strongly to E2 by inducing reporter activity, suggesting that both ERα and ERE are required for E2 to upregulate ElncRNA1 expression. Together, these findings indicate that E2 acts through its nuclear receptor ERα, which binds directly to ERE in the promoter region of ElncRNA1, thereby inducing transcriptional upregulation of ElncRNA1 expression. Additionally, we can conclude that E2 transcriptionally upregulates ElncRNA1 through the ERα-ERE pathway.

However, the role of this novel E2-upregulated lncRNA, ElncRNA1, remained to be determined. Accumulating evidence indicates that aberrantly expressed lncRNAs play oncogenic or tumour suppressor roles in human cancer. For example, HOTAIR promotes tumour growth in cervical cancer (38), and MEG3 inhibits cell proliferation in prostate cancer (39). As lncRNAs are emerging as key components of cancer progression, it was reasonable to hypothesize that ElncRNA1 may contribute to EOC progression. Our previous work showed that ElncRNA1 contributes to E2-induced EOC metastasis. However, the role of ElncRNA1 itself in EOC has not been determined, and an association between ElncRNA1 and aspects of EOC progression other than metastasis has not been demonstrated. In the present study, ElncRNA1 levels were clearly higher in EOC tissues than in noncancerous tissues, suggesting that ElncRNA1 may play a role in the aggressiveness of EOC. *In vitro*, ElncRNA1 knockdown suppressed cell proliferation and reduced the number of SKOV3 and CAOV3 colonies. Additionally, *in vivo* experiments confirmed that ElncRNA1 depletion inhibited tumour growth in nude mice. Taken together, these data suggest that ElncRNA1 plays an important role in promoting EOC growth and can function as an oncogene, thereby contributing to the proliferative effects of E2.

Previous study demonstrated that the key proliferation-related genes 'cyclin D1-CDK4/6', associated with E2/ER signaling pathway, promoted breast cancer proliferation (40). In the present study, we found that ElncRNA1 knockdown in SKOV3 and CAOV3 cells resulted in a significant decrease in CDK4, CDK6 and cyclin D1 protein levels instead of cyclin E and CDK2 levels, suggesting that ElncRNA1 regulates EOC cell proliferation at least in part by regulating 'cyclin D1-CDK4/6'. One limitation of this study is that we did not study the exact mechanism by which ElncRNA1

regulates cyclin D1-CDK4/6 in EOC growth. Further studies are required to explore the exact molecular mechanism of E2/ERα- ElncRNA1-'cyclin D1-CDK4/6' pathway.

In conclusion, our study provides the first evidence that E2 transcriptionally upregulates ElncRNA1 through the ERα-ERE pathway and that this novel E2-upregulated lncRNA has an oncogenic role in EOC growth. The addition of ElncRNA1 to the E2-ERα-ERE signalling pathway may provide greater insight into the oestrogenic effects on EOC progression from the perspective of lncRNA.

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