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# Long non-coding RNA LINP1 functions as an oncogene in endometrial cancer progression by regulating the PI3K/AKT signaling pathway

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**Abstract. – OBJECTIVE: Endometrial cancer (EC) accounts for about 6% of new cancer cases in female and about 3% of cancer-related deaths were caused by EC. The poor prognosis is mainly due to the distant spread and poor differentiation. In the current study, we want to figure out the role of long non-coding RNA (LncRNA) LINP1 in EC progression.**

**PATIENTS AND METHODS: The quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay was involved to access the expression level of LINP1 in EC cell lines and tissues. The Cell Counting Kit-8 (CCK-8) assay, colony formation assay, transwell and Matrigel assay were recruited to figure out the ability of LINP1 in cell proliferation and metastasis in EC. Subsequently, Western blotting was used to detect the expression level of PI3K/AKT in EC. Besides, we used the tumor formation assay *in vivo* to examine the ability of LINP1 in tumor formation *in vivo*.**

**RESULTS: LINP1 was proved to be up-regulated in EC cell lines and tissues by qRT-PCR assay. CCK-8 assay and colony formation assay were conducted and the results indicated that LINP1 over-expression can promote cell proliferation in EC *in vitro*. The data of transwell and Matrigel assays indicated that up-regulated LINP1 can facilitate cell migration and invasion. The results of Western blotting validated that LINP1 can activate PI3K/AKT signaling. Besides, the tumor formation assay verified that LINP1 can promote tumor formation *in vivo*.**

**CONCLUSIONS: Our research validated that LINP1 served as an oncogenic role in EC progression. The PI3K/AKT signaling pathway might be the underlying mechanism of EC progression. We hope our study can provide novel treatment targets and biomarkers in EC development and progression.**

*Key Words:*
LncRNA LINP1, Endometrial cancer, Proliferation, Metastasis, PI3K/AKT.

## Introduction

Endometrial cancer (EC) is a common malignancy type in the world and its prognosis is expressively related with the stage when diagnosed[1]. The incidence of EC is still increasing in the world and is a major cause of morbidity and mortality in women[2]. The poor prognosis was mainly due to the lack of efficient treatment strategies and accurate prognostic markers for EC[3]. Herein, in the current study, we carried out a series assays to prove that long non-coding RNA LINP1 might provide a new approach for finding a novel biomarkers and treatment targets for EC.

Long non-coding RNAs (LncRNAs) are a type of non-coding RNAs that cannot code protein and have a length of 200 nucleotides or more[4]. LncRNAs were proved to have multiple physiological functions such as cell proliferation, cell metastasis, cell apoptosis and cell cycle[5-7]. The essential role of lncRNAs has been reported[8-10] to exert their vital functions in various cancers including colorectal cancer, gastric cancer, ovarian cancer, etc. We conducted the current study to figure out the underlying mechanism of lncRNAs in EC progression.

In the current research, we used the qRT-PCR assay to examine the expression level of LINP1 in EC tissues and cell lines. The results indicated that LINP1 was up-regulated in EC. Subsequently, we employed the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto, Japan) and colony formation assay to access the ability of LINP1 on cell proliferation. It turned out that LINP1 can promote cell proliferation in EC. Additionally, LINP1 over-expression can accelerate cell migration and invasion by transwell assay and Matrigel assay. By detecting the expression level of PI3K,



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phosphorylation of PI3K, AKT, phosphorylation of AKT, we suggested that the PI3K/AKT signaling may be the underlying mechanism of LINP1 in EC progression. In tumorigenicity assay, the results elucidated that LINP1 can promote tumor formation *in vivo*. All the results presented indicated that LINP1 functioned as an oncogene in EC progression by regulating the PI3K/AKT signaling pathway.

## Patients and Methods

### Tissue Specimens

In the current research, 35 pairs of EC tissues and normal tissues were taken part in. All tissue specimens were obtained from patients who got surgery treatment in The First Affiliated Hospital of Anhui Medical University from 2016-2017. Tissue specimens were immediately put into liquid nitrogen. All tumor tissues were approved by pathological examination. This study was approved by the Ethics Committee of The First Affiliated Hospital of Anhui Medical University. Informed consents were signed from all participants before the study.

### Cell Lines

A total of 4 EC cell lines KLE, AN3CA, ECC-1 and endometrial fibroblast cell line T-HESC were obtained from the Shanghai Cell Bank (Shanghai, China). All cell lines were cultured by Roswell Park Memorial Institute-1640 medium (RPMI-1640; HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% $CO_2$.

### Cell Transfection

Lentivirus vector were constructed by GenePharma (Shanghai, China). Selected cell lines were transfected by lentiviral vectors for over-expressing or knocking down LINP1. All transfections were conducted following manufacturer's instructions. The transfection efficiency was detected by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) assay.

### Isolation of Total RNA and quantitative Real Time-Polymerase Chain Reaction

Total RNA of tissue specimens and cell lines was extracted through the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following standard protocol. All cDNAs were synthesized *via* Reverse Transcription Kit (TaKaRa, Otsu, Shiga, Japan) according to standard protocol. LncRNA-LINP1 expression level was assessed through SYBR Green Real Time-Polymerase Chain Reaction and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was taken as a normalization.

### Colony Formation

Cells ($1.0 \times 10^3$) was plated into the culture plates (60 mm) and cultured for 2 weeks. Cells on the plates were then washed by Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) twice and fixed in ice-cold 70% methanol for 15 min; Crystal Violet Staining Solution (Beyotime, Shanghai, China) was used to stain the cell colonies. All the colonies were subsequently photographed.

### Cell Counting Kit-8 Assay (CCK-8)

The Cell Counting Kit-8 assay was involved to examine the cell proliferation in EC. Transfected cells were plated into 96-well plates ($6 \times 10^3$/well) and then a CCK-8 solution (Beyotime, Shanghai, China; 10 μL/well) was used to stain cells for 2 hours at 37°C. The optical density (OD) value (450 nm) was then evaluated.

### Transwell Assay and Matrigel Assay

Transwell chambers and 24-wells plates were obtained from Corning (Lowell, MA, USA). $1.5 \times 10^5$ cells were suspended with serum-free medium (100 μL) and transferred to the upper chamber. In the Matrigel assay, cells were transferred to the Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) coated upper chamber. After 36 hours, the invasive cells were counted from images of five random fields using an inverted microscope (Olympus, Tokyo, Japan).

### Western Blot

Total protein was isolated by radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA) and Phenylmethanesulfonyl fluoride (PMSF). Protein lysates isolated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were then transferred to polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). The membrane was immunostained at 4°C by primary antibodies overnight. Primary rabbit antibodies used in the current study including anti-PI3K (Cell Signaling Technology, Danvers, MA, USA), anti-p-PI3K (Cell Signaling Tech-

nology, Danvers, MA, USA), anti-AKT (Cell Signaling Technology, Danvers, MA, USA), anti-p-AKT (Cell Signaling Technology, Danvers, MA, USA). Rabbit anti-GAPDH (Cell Signaling Technology, Danvers, MA, USA) was taken as a loading control. Protein relative expression level was determined by Image Lab software (NIH, Bethesda, MD, USA).

### Xenograft Model

This study was approved by the Animal Ethics Committee of Anhui Medical University Animal Center. Transfected KLE or ECC-1 cells ($7 \times 10^5$/mL) were injected into two flanks of nude mice (6 weeks old) subcutaneously. Tumors growth were monitored and recorded every week. The formula (volume= length × width$^2$ × 1/2) was used to calculate tumor volume. Tumors were extracted after 4 weeks.

### Statistics Analysis

All experiments in this work was performed at least three times independently. All data recorded were exhibited as mean ± standard deviation (SD). Student's unpaired *t*-test was used to undergo statistics analysis. In this study, *p*-values < 0.05 were considered statistically significant.

## Results

### Long Non-Coding RNA LINP1 Was Overexpressed in EC

For determining the expression level of LNIP1 in EC, we recruited the qRT-PCR assay. As shown in Figure 1A, LINP1 was up-regulated in tumor tissues when compared with normal tissues. Consistently, we examined the expression level of LINP1 in EC cell lines. The data showed that EC cell lines had a relatively higher expression level of LINP1 in comparison with the endometrial fibroblast cell line (Figure 1B). Thus, the results indicated that LINP1 was over-expressed in EC. Besides, we conducted cell transfection in selected EC cell lines and the endometrial fibroblast cell line. The transfection efficiency was accessed by qRT-PCR. As shown in Figure 1C, LINP1 over-expression group had a relatively higher

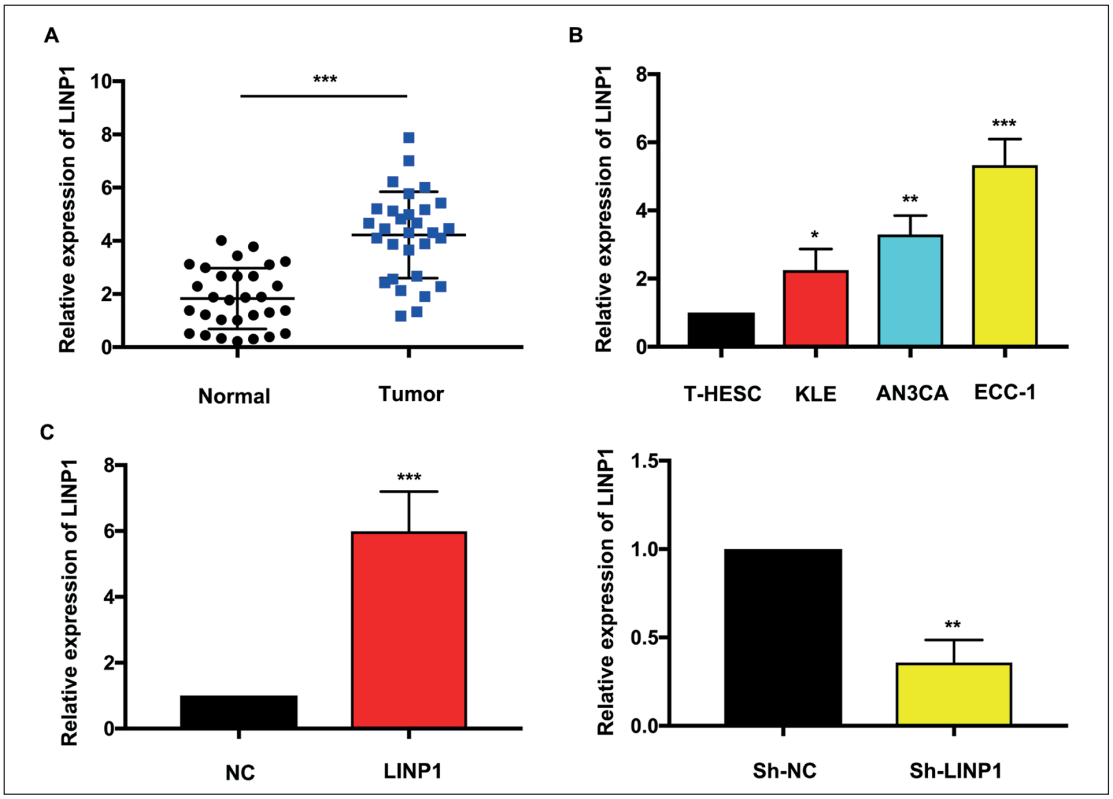


**Figure 1.** LINP1 was up-regulated in EC cell lines and tissues. ***A,*** The expression level of LINP1 in EC tissues and normal tissues. ***B,*** Analysis of LINP1 expression level in EC cell lines. ***C,*** Transfection efficiency was evaluated by qRT-PCR. Data are presented as the mean ± SD of three independent experiments. **p*<0.05, ***p*<0.01, ****p*<0.001.

expression level of LINP1 while the oppositive result was emerged in the Sh-LINP1 group.

### Over-Expression LINP1 can Promote Cell Proliferation in EC Cell Lines

To figure out the function of LINP1 in cell proliferation, we conducted cell proliferation assays including CCK-8 assay and colony formation assay. As Figure 2A showed, over-expressed LINP1 expressively increased the OD value in EC cell lines while LINP1 down-expression had a relatively lower OD value when compared with the control group. Similarly, the colonies generated in LINP1 up-regulated group was simply more than the control group. The LINP1 down-expression group generated less colonies in comparison with the control group (Figure 2B). All the results suggested that over-expression of LINP1 can promote cell proliferation in EC cell lines.

### LINP1 Over-Expression Induced Cell Metastasis in EC Cell Lines

The transwell assay and Matrigel assay were involved in this study for investigating the ability of LINP1 on cell migration and invasion. As Figure 3A showed, over-expression of LINP1

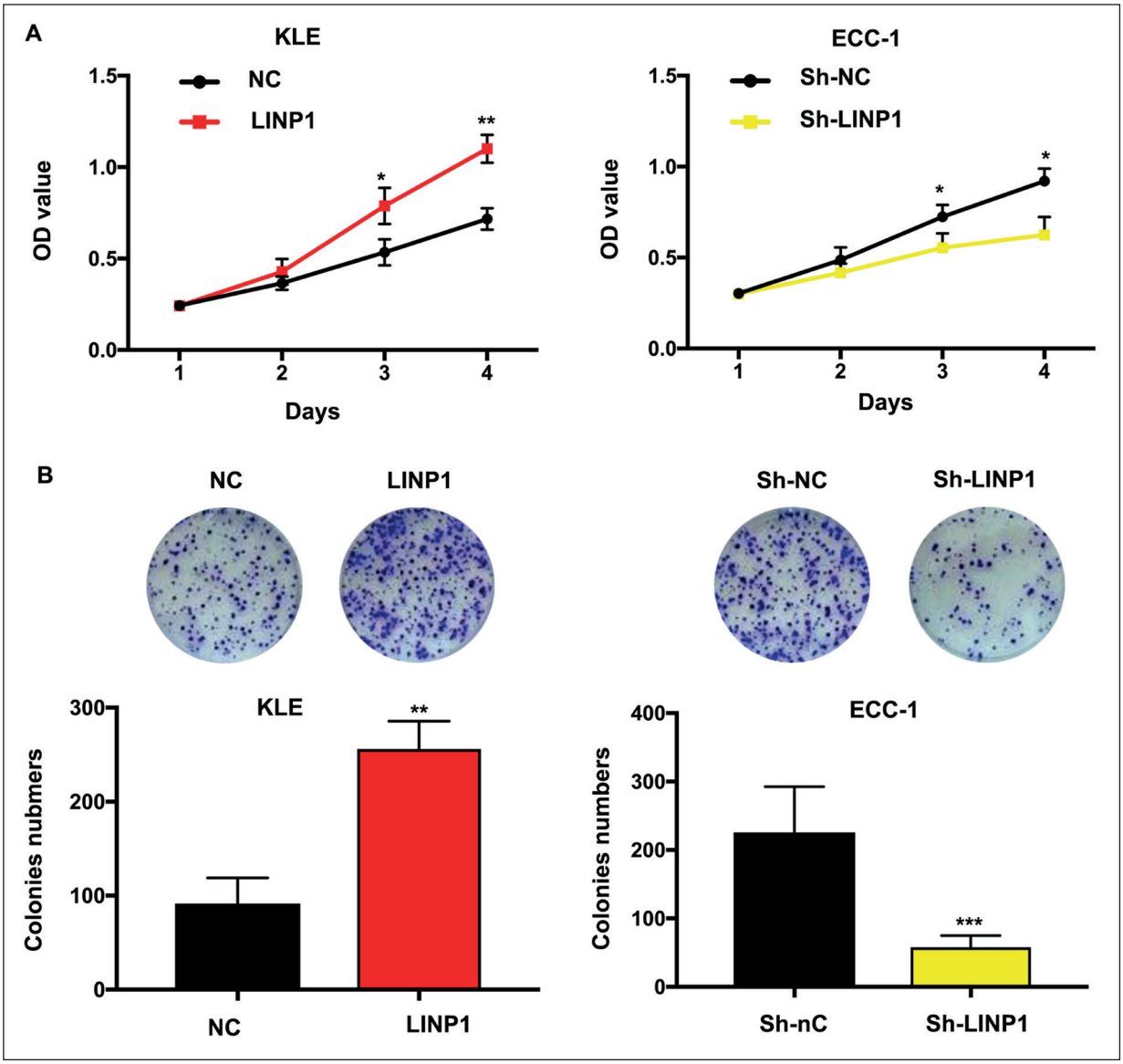


**Figure 2.** LINP1 promoted cell proliferation in EC. ***A,*** The cell proliferation ability was determined by the CCK-8 assay. ***B,*** The colony formation assay was involved to examine the effects on cell proliferation. Data are presented as the mean ± SD of three independent experiments. *$p$< 0.05, **$p$<0.01, ***$p$<0.001.

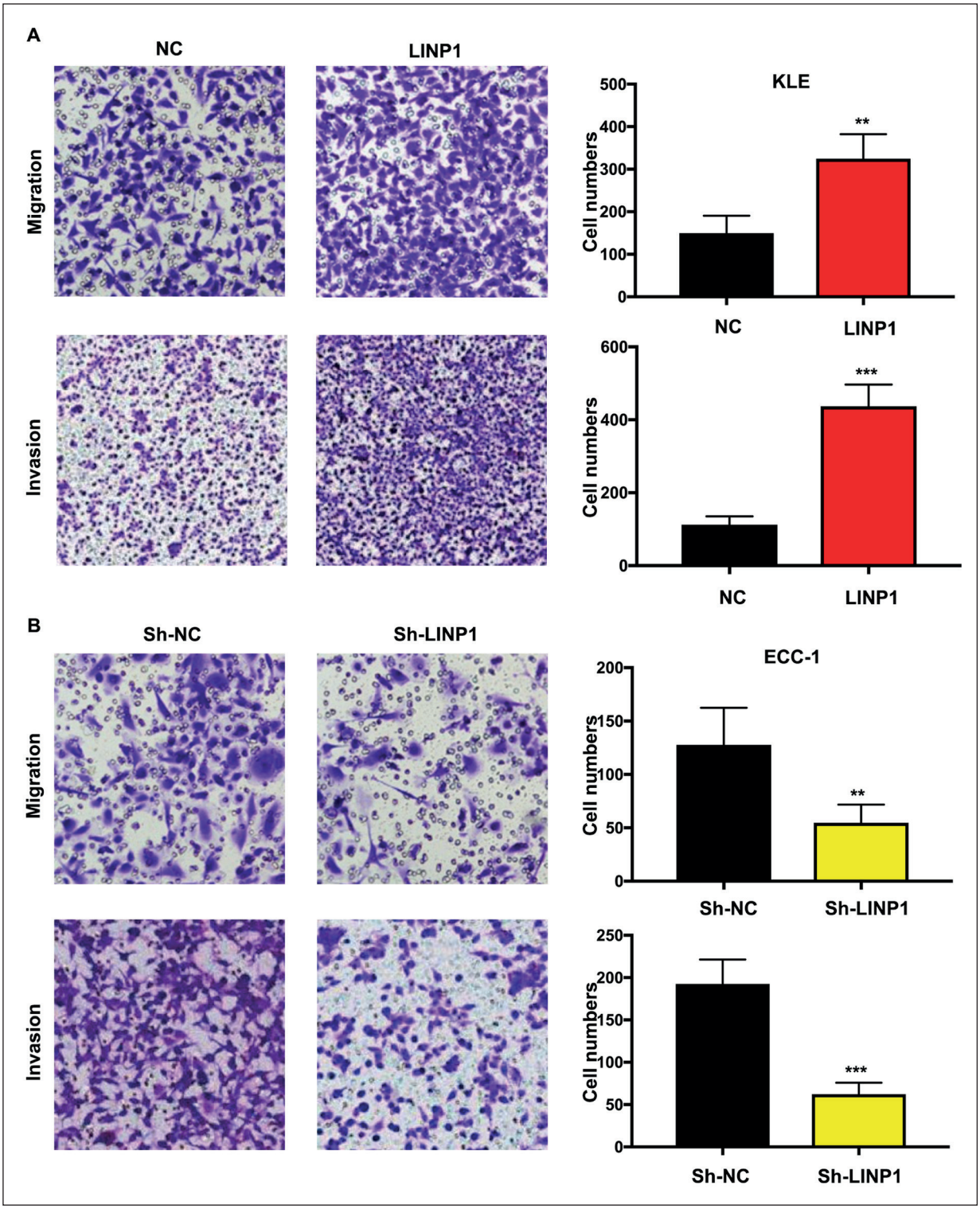


**Figure 3.** Up-regulated LINP1 can facilitate cell migration and invasion. ***A,*** The transwell assay and Matrigel assay were recruited to examine the ability of LINP1 in cell migration and invasion in transfected KLE cell line (magnification: 40×). ***B,*** The transwell assay and Matrigel assay were recruited to examine the ability of LINP1 in cell migration and invasion in transfected ECC-1 cell line (magnification: 40×). Data are presented as the mean ± SD of three independent experiments. $^{**}p<0.01$.

group promoted cell migration and had more invasive cells. Conversely, down-regulated LINP1 induced less cell migration and had less invasive cells (Figure 3B). LINP1 over-expression can lead to cell metastasis in EC cell lines.

### *The Underlying Mechanism of LINP1 in EC Progression*

For elucidating the molecular mechanism underlying LINP1 in EC progression, we wonder if PI3K/AKT take part in EC progression. Thus, we recruited Western blotting assay to detect PI3K, phosphorylation of PI3K, AKT, phosphorylation of AKT, GAPDH. As shown in Figure 4A and 4B, over-expression of LINP1 increased the phosphorylation of PI3K and phosphorylation of AKT while down-regulated LINP1 lead to less phosphorylation of PI3K and AKT. We considered that the PI3K/AKT pathway may be the underlying mechanism of LINP1 in EC progression.

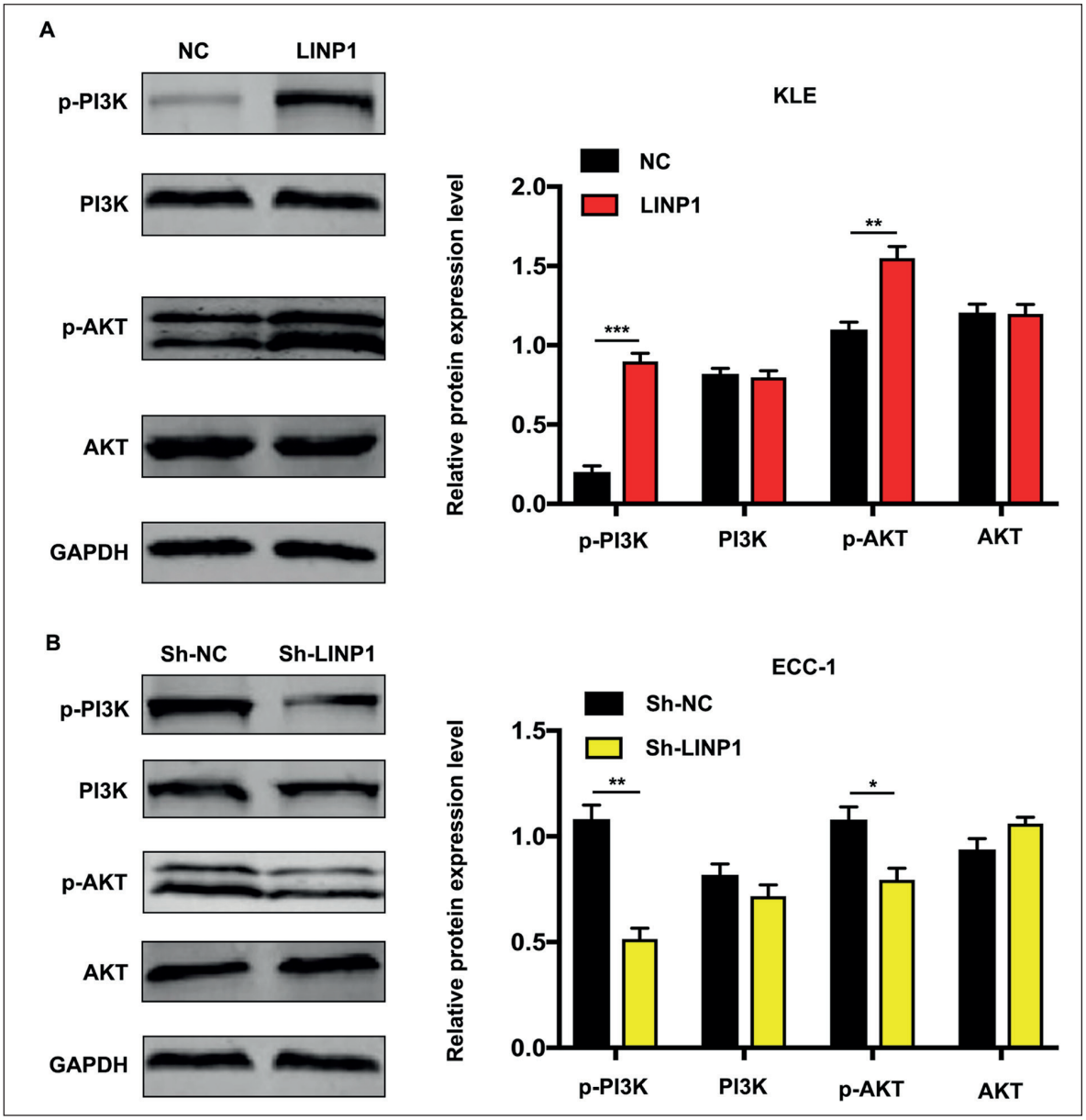


**Figure 4.** Over-expression of LINP1 can activate the PI3K/AKT signaling pathway. ***A,*** PI3K, p-PI3K, AKT, p-AKT protein expression levels were examined in over-expression LINP1 group. ***B,*** PI3K, p-PI3K, AKT, p-AKT protein expression levels were examined in down-expression LINP1 group. Data are presented as the mean ± SD of three independent experiments. *$p$<0.05, **$p$<0.01, ***$p$<0.001.

### *LINP1 Led to Tumor Formation In Vivo*

To figure out the ability of LINP1 in tumor formation *in vivo*, we conducted tumorigenicity assay. As Figure 5A showed, LINP1 up-regulated group had larger size in tumor volume when compared with the control group. The tumor size in down-expressed LINP1 group lead to smaller tumor volume in comparison with the control group. Besides, we examined the expression level of LINP1 in generated tumor tissues. The results showed that the tumor tissues of LINP1 over-expression group had relatively higher expression level of LINP1 and the LINP1 down-regulated group had lower expression level of LINP1 (Figure 5B). All data indicated that LINP1 led to tumor formation *in vivo*.

## Discussion

EC accounts for about 6% of new malignancies cases in female and about 3% of cancer related deaths were caused by EC[11]. The prognosis of early-stage EC patients was favorable due to the symptom of bleeding[12]. However, the prognosis of EC patients with advanced stage was still poor on account of distant spread, poor differentiation[11]. Hence, it is meaningful to seek for novel therapeutic targets and biomarkers of EC. LncRNAs have been studied for years and their multiple physiological functions were proved to have great effects on cancer progression. In this work, we carried out assays both *in vitro* and *in vivo* to prove that lncRNA LINP1 played an important role in EC progression.

LncRNAs were found to have diverse functions in cancer progression and exert a vital role in tumorigenicity[4]. The multiple physiological functions of lncRNAs were mainly obtained by post-transcriptional regulation, transcriptional regulation or chromatin modification[13]. Various lncRNAs have been reported to exhibit diverse functions in EC development and progression[14,15]. However, the underlying molecular mechanism of EC progression re-

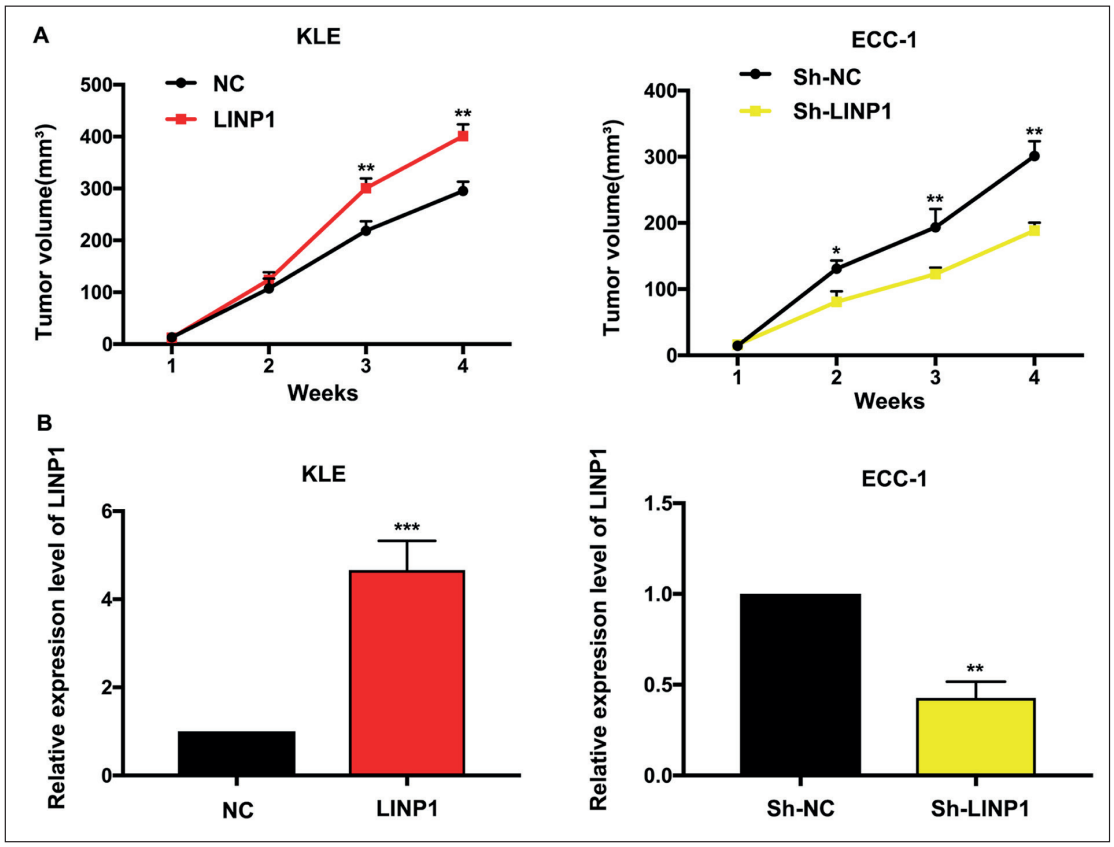


**Figure 5.** Up-regulated LINP1 can facilitate tumor formation *in vivo*. ***A,*** After tumor extraction, tumor volume was calculated respectively and made into a graph. ***B,*** The relative expression of LINP1 in tumors were examined by qRT-PCR. Data are presented as the mean ± SD of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

mains unknown. LncRNA LINP1 has been reported to regulate repair of DNA double-strand breaks in triple-negative breast cancer[16]. TGF-β/SMAD4-regulated lncRNA-LINP1 was proved to inhibit epithelial-mesenchymal transition in lung cancer[17]. Nevertheless, the biological function of LINP1 in EC has rarely been studied. In the current work, we examined the expression level of LINP1 in EC cell lines and tissues and the results showed that LINP1 was up-regulated in EC. Subsequently, we used CCK-8 assay, colony formation assay, transwell and Matrigel assay to prove that LINP1 can promote cell proliferation and metastasis in EC. Besides, the tumorigenesis assay *in vivo* elucidated that LINP1 can facilitate tumor formation *in vivo*. We validated that LINP1 functioned as an oncogenic role in EC development and progression.

To figure out the underlying mechanism of the oncogenic role of LINP1 in EC, we detected the expression level of PI3K/AKT signaling. PI3K/AKT was proved to take part in various cellular progressions including cell proliferation, cell metastasis, cell apoptosis and cell cycle[18,19]. It is also reported[20-22] that PI3K/AKT exerted their functions in breast cancer, ovarian cancer including endometrial cancer. Hence, we wonder if PI3K/AKT is the underlying mechanism of the oncogenic role of LINP1 in EC. As the results showed, over-expression of LINP1 can activate the phosphorylation of PI3K and AKT. Hence, we considered that LINP1 may function as an oncogenic role in EC by regulating the PI3K/AKT signaling pathway. All the results indicated that LINP1 served as an oncogenic role in EC by regulating the PI3K/AKT signaling pathway.

## Conclusions

Our research presented abundant evidence to illustrate that LINP1 functioned as an oncogene in EC progression. The PI3K/AKT signaling pathway might be the underlying mechanism of EC progression. We hope our study can provide novel treatment targets and biomarkers in EC development and progression.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

## References


1) Sorosky JI. Endometrial cancer. Obstet Gynecol 2012; 120: 383-397.
2) Lee YC, Lheureux S, Oza AM. Treatment strategies for endometrial cancer: current practice and perspective. Curr Opin Obstet Gynecol 2017; 29: 47-58.
3) Binder PS, Mutch DG. Update on prognostic markers for endometrial cancer. Womens Health (Lond) 2014; 10: 277-288.
4) Renganathan A, Felley-Bosco E. Long noncoding RNAs in cancer and therapeutic potential. Adv Exp Med Biol 2017; 1008: 199-222.
5) Wei GH, Wang X. lncRNA MEG3 inhibit proliferation and metastasis of gastric cancer via p53 signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 3850-3856.
6) Wang MW, Liu J, Liu Q, Xu QH, Li TF, Jin S, Xia TS. LncRNA SNHG7 promotes the proliferation and inhibits apoptosis of gastric cancer cells by repressing the P15 and P16 expression. Eur Rev Med Pharmacol Sci 2017; 21: 4613-4622.
7) Liu Y, Zhao J, Zhang W, Gan J, Hu C, Huang G, Zhang Y. lncRNA GAS5 enhances G1 cell cycle arrest via binding to YBX1 to regulate p21 expression in stomach cancer. Sci Rep 2015; 5: 10159.
8) Wang JZ, Xu CL, Wu H, Shen SJ. LncRNA SNHG12 promotes cell growth and inhibits cell apoptosis in colorectal cancer cells. Braz J Med Biol Res 2017; 50: e6079.
9) Liu Z, Chen Z, Fan R, Jiang B, Chen X, Chen Q, Nie F, Lu K, Sun M. Over-expressed long noncoding RNA HOXA11-AS promotes cell cycle progression and metastasis in gastric cancer. Mol Cancer 2017; 16: 82.
10) Li X, Wang X, Mao L, Zhao S, Wei H. LncRNA TP73AS1 predicts poor prognosis and promotes cell proliferation in ovarian cancer via cell cycle and apoptosis regulation. Mol Med Rep 2018; 18: 516-522.
11) Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013; 63: 11-30.
12) Amant F, Mirza MR, Creutzberg CL. Cancer of the corpus uteri. Int J Gynaecol Obstet 2012; 119 Suppl 2: S110-S117.
13) Huarte M. The emerging role of lncRNAs in cancer. Nat Med 2015; 21: 1253-1261.
14) Xie P, Cao H, Li Y, Wang J, Cui Z. Knockdown of lncRNA CCAT2 inhibits endometrial cancer cells growth and metastasis via sponging miR-216b. Cancer Biomark 2017; 21: 123-133.
15) Sun MY, Zhu JY, Zhang CY, Zhang M, Song YN, Rahman K, Zhang LJ, Zhang H. Autophagy regulated by lncRNA HOTAIR contributes to the cisplatin-induced resistance in endometrial cancer cells. Biotechnol Lett 2017; 39: 1477-1484.
16) Zhang Y, He Q, Hu Z, Feng Y, Fan L, Tang Z, Yuan J, Shan W, Li C, Hu X, Tanyi JL, Fan Y, Huang Q, Montone K, Dang CV, Zhang L. Long noncoding RNA

LINP1 regulates repair of DNA double-strand breaks in triple-negative breast cancer. Nat Struct Mol Biol 2016; 23: 522-530.

17) Zhang C, Hao Y, Wang Y, Xu J, Teng Y, Yang X. TGF-beta/SMAD4-regulated lncRNA-LINP1 inhibits epithelial-mesenchymal transition in lung cancer. Int J Biol Sci 2018; 14: 1715-1723.

18) Zhou Y, Li S, Li J, Wang D, Li Q. Effect of microRNA-135a on cell proliferation, migration, invasion, apoptosis and tumor angiogenesis through the IGF-1/PI3K/Akt signaling pathway in non-small cell lung cancer. Cell Physiol Biochem 2017; 42: 1431-1446.

19) Qu Y, Hao C, Xu J, Cheng Z, Wang W, Liu H. ILK promotes cell proliferation in breast cancer cells by activating the PI3K/Akt pathway. Mol Med Rep 2017; 16: 5036-5042.

20) Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res 2012; 18: 5856-5864.

21) Jin Y, Feng SJ, Qiu S, Shao N, Zheng JH. LncRNA MALAT1 promotes proliferation and metastasis in epithelial ovarian cancer via the PI3K-AKT pathway. Eur Rev Med Pharmacol Sci 2017; 21: 3176-3184.

22) Lee JH, Son YH, Kwon YW, Park SY, Koo BS, Jung SH. Anticancer effects of a Korean herbal medicine formula (H9) via AMPK and HER2-PI3K/Akt signaling in breast cancer cells. Phytother Res 2017; 31: 1765-1775.