# Nuclear estrogen receptor-mediated Notch signaling and GPR30-mediated PI3K/AKT signaling in the regulation of endometrial cancer cell proliferation

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**Abstract.** To elucidate the mechanisms of nuclear estrogen receptor (ER)-mediated and G protein-coupled receptor 30 (GPR30)-mediated signaling in the regulation of proliferation in ER-positive and ER-negative endometrial cancer cells, two human endometrial carcinoma cell lines, Ishikawa (ER-positive) and KLE (ER-negative), were used. PCR and Western blot analyses were used to determine the effects of estrogen stimulation on the activation of Notch and GPR30-PI3K/AKT signaling. Cell growth was investigated using MTT assays. Overexpression of ER in ER-negative cells was achieved by plasmid transfection and was used to investigate the effects on cellular growth and Notch signaling. GPR30-mediated signaling was evaluated using siRNA interference. Estrogen stimulated cell proliferation in both cell lines, it activated Notch signaling in ER-positive Ishikawa cells, but not in ER-negative KLE cells. Blockade of this signaling by a Notch inhibitor resulted in partial arrest of estrogen-induced cell proliferation in Ishikawa cells. Overexpression of ER in KLE cells restored estrogen-enhanced Notch signaling and further promoted cell growth. GPR30, as a new G-protein-coupled estrogen receptor, was detected in both cell lines, but was stronger in ER-negative KLE cells. Depletion of GPR30 in KLE cells abolished estrogen-induced PI3K/AKT signaling activation and resulted in inhibition of cell proliferation. Conclusively, regulation of proliferation in nuclear ER-positive endometrial cancer cells is mediated by both ER-Notch signaling and GPR30-PI3K/AKT signaling, whereas only the latter pathway is involved in the regulation of growth in nuclear ER-negative endometrial cancer cells.

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*Key words:* Notch, GPR30, endometrial cancer, estrogen, proliferation

## Introduction

Endometrial cancer is the most common gynecological malignancy worldwide and represents the eighth leading cause of cancer-related deaths in women (1-3). Most endometrial adenocarcinomas are characterized by positive nuclear estrogen receptor (ER) expression and responsiveness to hormone stimulation. Increasing evidence indicates that prolonged estrogen exposure is associated with initiation of type 1 endometrioid cancers. Estrogen exposure results in an overall physiological response within several hours by a genomic mechanism which depends on estrogen binding to the nuclear ER resulting in mRNA transcription and protein synthesis of target genes. Despite extensive research focused on the mechanism of estrogen-driven endometrial cancer development, the downstream signaling events mediated by nuclear ER remain to be clarified.

Aberrant Notch signaling has been documented in various cancer types and has been associated with tumorigenesis (4-6). Notch signaling is an evolutionarily conserved pathway encompassing a family of transmembrane receptors composed of an extracellular subunit linked to transmembrane and intracellular subunits via heterodimerization domains (7). The interaction between Notch receptors and their ligands results in proteolytic events that release an active form of the Notch intracellular domain (NICD) from the plasma membrane. This translocates to the nucleus and functions as a transcriptional activator to enhance the expression of target genes associated with cell proliferation (8,9). Notch1 down-regulation has been reported to result in growth arrest and apoptosis of pancreatic cancer cells (10). Moreover, the γ-secretase inhibitor N-[N-(3, 5-difluorophenacety)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), inhibits proliferation and induces apoptosis through Notch signaling in a variety of cancer cell types, including ovarian cancer (11). However, the involvement of Notch signaling in the regulation of estrogen-induced endometrial

cancer cell proliferation remains to be elucidated. Furthermore, it is unclear whether this signaling is mediated by nuclear ER.

The results of previous studies have led to the commonly held belief that estrogen-induced endometrial cancer cell expansion is mediated by nuclear ER. However, recent studies have confirmed that GPR30, a member of the seven-transmembrane GPCR family, is involved in mediating rapid and transcription-independent estrogen signaling in various cell types as a transmembrane ER (12). Overexpression of GPR30 has been demonstrated in endometrial carcinomas with an expression profile that is associated with pathological grade (13). Furthermore, GPR30 was found to be involved in regulation of cell proliferation and invasive activity. However, it is not clear whether estrogen regulates cellular growth in ER-positive and negative cells through the same or different signaling pathways.

This study aimed to investigate the signaling pathways involved in the regulation of cell proliferation in nuclear ER-positive and ER-negative endometrial cancer cells. Data demonstrated that estrogen regulated cell growth in nuclear ER-negative endometrial cancer cells predominantly through GPR30-PI3K/AKT signaling, while, in nuclear ER-positive cells, Notch signaling mediated by ER was also involved.

## Materials and methods

*Reagents*. The antibodies against Notch1, GAPDH, pAKT, and AKT were obtained from Cell Signaling Technology. The antibody against GPR30 was obtained from Abcam company (Abcam, Cambridge Science Park, Cambridge, UK). MTT, β-estradiol ($E_2$), ICI 182,780 (ICI) and LY294002 were purchased from Sigma.

*Cell lines and cell culture*. Two established human endometrial carcinoma cell lines, Ishikawa and KLE, obtained from the American Type Culture Collection (Manassas, VA), were used in this study. The estrogen-responsive Ishikawa cell line was derived from a well-differentiated endometrioid carcinoma, known to contain nuclear estrogen receptors, whereas KLE cells do not express the nuclear estrogen receptor. Both cell lines were maintained in Dulbecco's modified Eagle's Medium (DMEM) in the presence of 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, sodium pyruvate and L-glutamine in a humidified atmosphere of 5% $CO_2$, at 37˚C.

*MTT assay*. To determine the proliferative activity, Ishikawa or KLE cells were seeded in 96-well plates at a density of 2,000 cells/well. After 24 h culture, the medium was replaced with serum-free DMEM and starved for a further 24 h. To address the effect of $E_2$ on cell proliferation, various concentrations of $E_2$ were used to treat both cell lines for another 48 h. In order to determine the effects of ICI, LY294002 or DAPT on cellular growth, cells were pre-treated with these inhibitors for 1 h, respectively, following stimulation with 1 nM $E_2$ or PBS for 48 h. Similarly, prior to 1 nM $E_2$ simulation, cells were transiently transfected with ER plasmid or GRP30 siRNA to investigate these factors' effect on cell proliferation. Cells were then stimulated for 48 h. MTT solution (20 $\mu$l of 5 mg/ml in PBS) was added to the culture for 4 h at 37˚C. Formazan was dissolved by the addition of dimethyl sulfoxide (DMSO) and absorbance at 570 nm was measured to determine cell viability.

Table I. Sequences of primers used for amplification of target genes.

| Gene | Primer nucleotide sequence |
|---|---|
| Notch1 | Forward: 5'-GCCGTCATCTCCGACTTCATC-3' |
| | Reverse: 5'-GGTGTCTCCTCCCTGTTG TTCTG-3' |
| ER | Forward: 5'-TCCAACTGCATTTCCTTTCC-3' |
| | Reverse: 5'-TTGGAACATGGCAGCATTTA-3' |
| GPR30 | Forward: 5'-AGTCGGATGTGAGGTTCAG-3' |
| | Reverse: 5'-TCTGTGTGAGGAGTGCAAG-3' |
| GAPDH | Forward: 5'-AACGGATTTGGTCGTATTG-3' |
| | Reverse: 5'-GGAAGATGGTGATGGGATT-3' |

*Plasmid transfections and hormone administration*. The pcDNA-ER plasmid and pcDNA empty vector were generous gifts from Dr Guiqiang Du (Shanghai Jiao Tong University, Shanghai, China). After serum starvation for 24 h, KLE cells were transfected with the plasmid using Lipofectamine™ 2000 according to the protocol provided by the manufacturer. The transfection efficiency was determined by RT-PCR of the ER gene. Subsequently, ER plasmid-transfected cells and control cells were treated with 1 nM $E_2$ for a further 48 h before investigation of Notch signaling by Western blot analysis and cell proliferation by the MTT assay.

*Small interfering RNA (siRNA) transfection and hormone stimulation*. GPR30 knockdown was performed in KLE cells seeded at a density of $1x10^4$ cells/well in a 6-well plate. Cells were serum-starved for 24 h prior to transfection with 50 nM SMARTpool GPR30 small interfering RNA (siRNA) (Dharmacon Research, Inc.) in the presence of Hiperfect transfection reagent (Dharmacon Research, Inc.) for 24 h. The siCon siRNA served as a transfection control. siRNA-treated and untreated KLE cells were exposed to 1 nM $E_2$ for a further 48 h before being collected for analysis.

*RNA extraction, RT-PCR and real-time PCR*. Total RNA was extracted from the cells after the indicated treatment by using the TRIzol reagent followed by isopropanol precipitation according to the instructions provided by the manufacturer. Total RNA (2 $\mu$g) was reverse-transcribed into cDNA in a 20-$\mu$l reaction according to the protocol provided with the PrimeScript™ RT reagent kit (Takara, Kyoto, Japan). The primers used for specific amplification of target genes (Table I) were synthesized by Invitrogen (Shanghai, China). PCR reactions were carried out under the following conditions: 95˚C for 10 min, 35 cycles at 95˚C for 15 sec, and (Notch) 60˚C/(GAPDH) 58˚C/(ER/GPR30) 55˚C for 30 sec and 72˚C for 30 sec. Real-time PCR was carried out using similar reaction conditions. All PCR experiments were performed in triplicate and the results are presented as a mean value for the determination of mRNA expression levels. GAPDH served as a loading control.

*Western blot analysis*. Treated cells were collected and lysed in ice-cold cell lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM

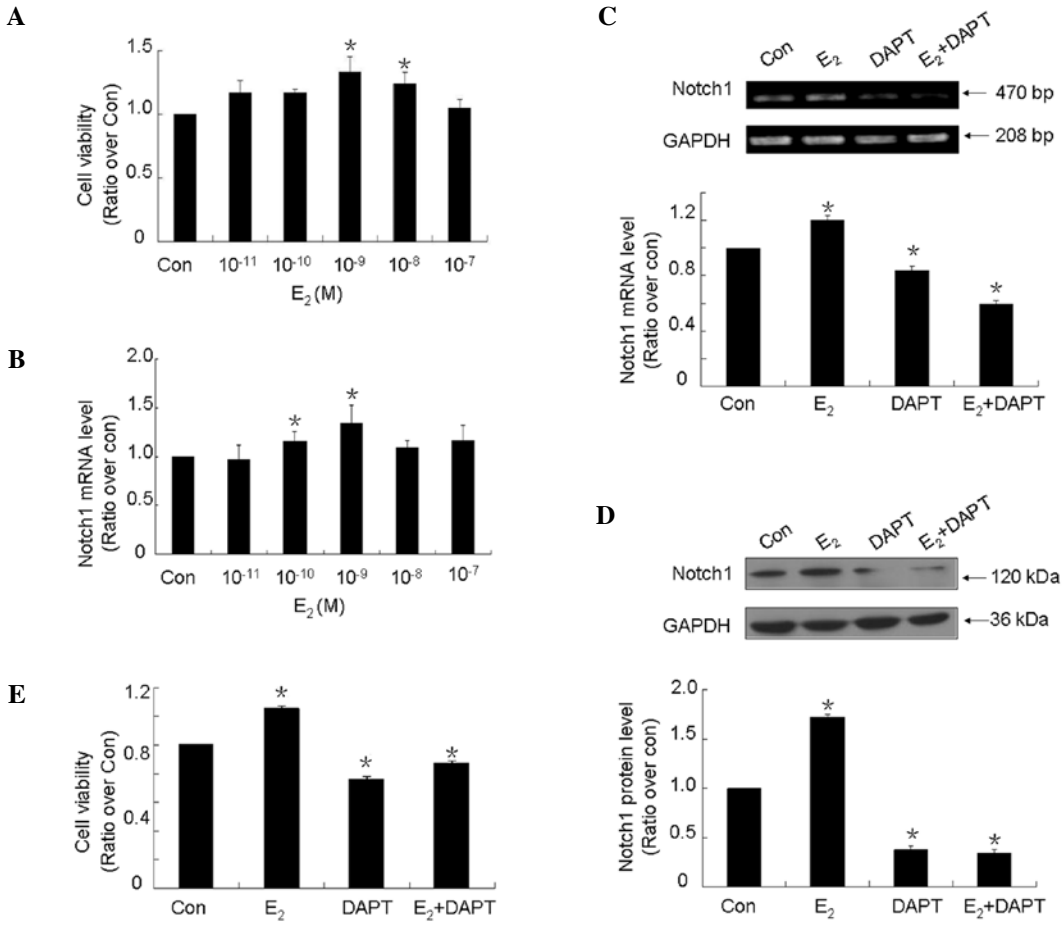


Figure 1. Estrogen enhances nuclear ER-positive endometrial cancer cell expansion through Notch signaling. (A) Cell proliferation following $E_2$ treatment with the indicated concentrations was assessed using MTT assays. Values are the mean ± SD of six experiments. (B) The effect of $E_2$ on Notch1 mRNA was monitored by real-time PCR. DAPT-mediated (10 $\mu$M) blockade of Notch1 signaling attenuated $E_2$-induced (1 nM) Notch1 mRNA (C) and protein (D) expression. (E) Inhibition of Notch signaling on $E_2$-induced cell proliferation was determined by the MTT assay. *Statistically significant difference compared with the control (Con).

NaCl, 30.02% N-acetyl-neuraminic acid (NaN), 0.1% SDS, 0.1% NP-40, 0.5% sodium taurodeoxycholate (NaTDC) and 1 mM EDTA] for protein extraction. Cell lysates were centrifuged at 12,000 rpm for 10 min at 4˚C. Protein concentrations were determined using a BCA protein assay kit (Thermo Scientific, Waltham, MA). Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% skimmed milk for 1 h and incubated overnight with the primary antibodies, followed by 1 h of incubation with the appropriate secondary antibody. Proteins were detected using an enhanced chemiluminescence reagent.

*Data analysis*. Data are presented as the mean ± standard deviation (SD). The Student's t-test was used to analyze the differences of cell proliferation, various target genes or protein expressions. A two-sided test with p-values <0.05 was considered statistically significant.

## Results

*Notch signaling in regulation of nuclear ER-positive endometrial cancer cell proliferation*. Estrogen was observed to promote Ishikawa cell proliferation and to enhance Notch1 expression in a dose-dependent manner with the maximum effects observed at 1 nM $E_2$ (Fig. 1A and B). RT-PCR and Western blot assays confirmed that 10 $\mu$M N-[N-(3, 5-difluorophenacety)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a potent γ-secretase inhibitor, significantly reduced the expression of Notch1. Moreover, $E_2$-induced Notch1 expression was blocked by DAPT treatment (Fig. 1C and D). The requirement for Notch pathway activation in Ishikawa cell growth and survival was confirmed by the MTT assay which revealed that 1 nM $E_2$ induced cellular growth which was partially abolished by 10 $\mu$M DAPT pre-treatment, whereas DAPT treatment alone resulted in more significant growth inhibition (Fig. 1E).

*Inhibition of nuclear ER partially abolishes estrogen-induced proliferation mediated by Notch signaling*. The role of nuclear ER in Notch signaling mediated estrogen-induced proliferation of Ishikawa cells was investigated using the nuclear ER inhibitor, ICI 182,780 (ICI). The 1 nM $E_2$-induced Notch1 expression was potently attenuated by ICI (10 $\mu$M) (Fig. 2A). Further investigation demonstrated that ICI treatment induced Ishikawa cell growth arrest. Moreover, the growth induced by $E_2$ was significantly inhibited by ICI, although the inhibition ratio to cell proliferation was not more potent than the blocking effect of Notch1 protein by ICI (Fig. 2B).

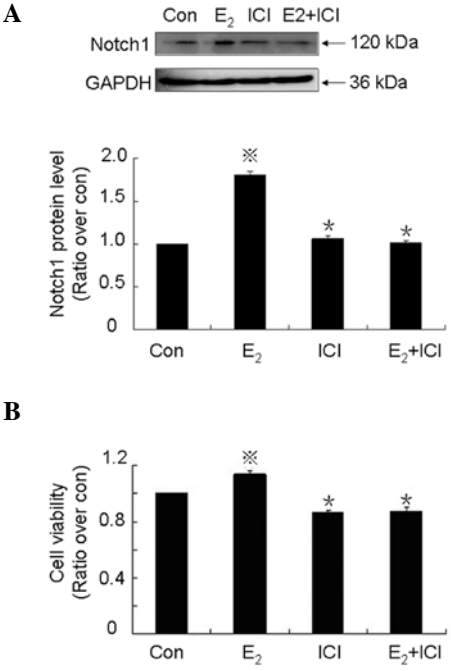


Figure 2. Inhibition of ER results in blockade of Notch signaling and induces Ishikawa cell proliferation arrest; 10 $\mu$M ICI treatment reduced $E_2$-induced Notch1 expression (A) and attenuated cellular growth (B). GAPDH served as a loading control (Con). MTT assays were performed six times. ※Statistically significant differences compared with the control (In A, p=0.028; In B, p=0.048); *p<0.05 when compared with the $E_2$-treated group.

*Notch signaling in estrogen induced-proliferation of nuclear ER-negative endometrial cancer cells*. The estrogen-responsiveness of the nuclear ER-negative KLE endometrial cancer cell line remains a controversial issue. In this study, estrogen enhanced KLE cellular growth in a dose-dependent manner with maximal proliferative activity stimulated by 1 nM $E_2$ (Fig. 3A). However, in contrast to the Ishikawa cell line, treatment with various concentrations of $E_2$ had no obvious effect on Notch1 mRNA levels in KLE cells (Fig. 3B). RT-PCR and Western blot assay confirmed that Notch1 expression was not induced by 1 nM $E_2$ stimulation of nuclear ER-negative KLE cells (Fig. 3C and D). Although we observed a significant inhibitory effect of DAPT on cellular growth, only a slight inhibitory effect of DAPT on $E_2$-induced cell proliferation activity was observed (Fig. 3E).

*Restoration of estrogen-induced Notch1 expression and cell growth by overexpression of nuclear ER*. Transient transfection of nuclear ER in KLE cells resulted in overexpression of nuclear ER compared with the control group and the group transfected with the empty vector (Fig. 4A). Western blot analysis revealed that overexpression of nuclear ER plus $E_2$ stimulation significantly up-regulated Notch1 protein levels >4-fold compared with $E_2$-treated cells. Furthermore, transfection with the nuclear ER plasmid restored the $E_2$-induced activation of the Notch signaling pathway (Fig. 4B). Subsequent

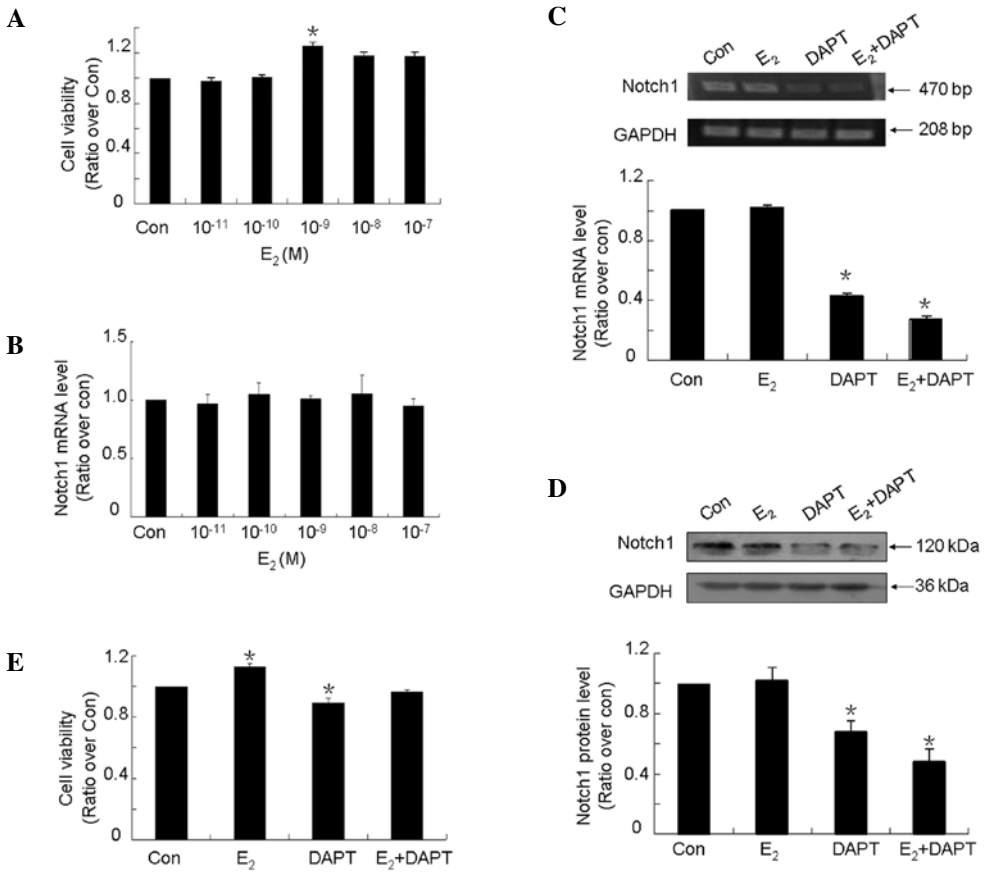


Figure 3. Estrogen-induced proliferation in nuclear ER-negative endometrial cancer cell is not mediated by Notch signaling. (A) KLE cell proliferation following $E_2$ treatment with the indicated concentrations was assessed using MTT assays. Values are the mean ± SD of six experiments. (B) The effect of various concentrations of $E_2$ on Notch1 mRNA was monitored by real-time PCR. DAPT-mediated (10 $\mu$M) blockade of Notch1 signaling in the presence and absence of $E_2$ (1 nM) attenuated Notch1 mRNA (C) and protein (D) expression. (E) Inhibition of Notch signaling on $E_2$-induced cell proliferation was determined by the MTT assay. *Statistically significant differences compared with the control (Con).

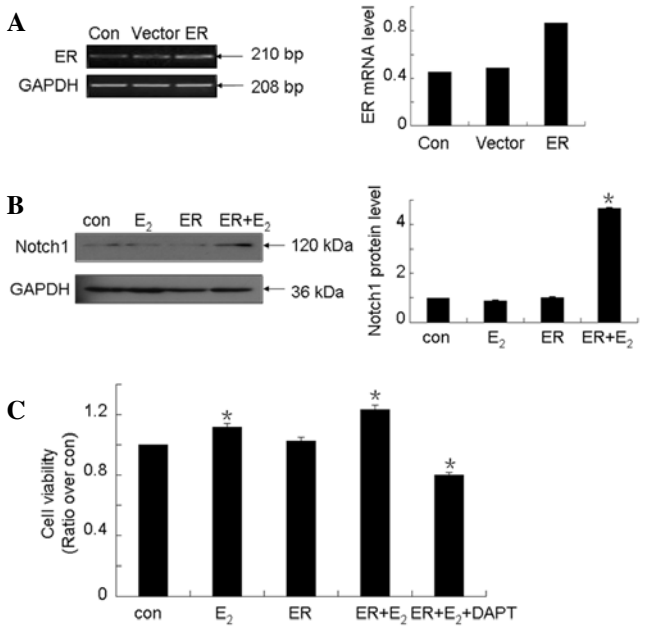


Figure 4. Overexpressing ER restores estrogen regulated cell proliferation through Notch signaling in ER-negative endometrial cancer cells. (A) ER mRNA was determined by RT-PCR after transfection. (B) Western blot analysis of $E_2$-enhanced (1 nM) Notch1 expression following ER plasmid transfection (*p<0.001). GAPDH was used as a loading control (Con). (C) $E_2$ (1 nM)-induced expansion of ER-transfected KLE cells was inhibited by DAPT treatment (10 $\mu$M). This experiment was repeated six times. *Statistically significant compared with the control (Con).

investigation confirmed that transfection with the ER plasmid plus $E_2$ stimulation promoted greater KLE cell expansion than that observed with $E_2$ treatment alone. Moreover, this increase in proliferative activity was abolished by DAPT treatment (Fig. 4C).

*GPR30-PI3K/AKT signaling in regulation of proliferation in nuclear ER-negative cells*. Expression of GPR30 and downstream signaling molecules was investigated by Western blot analysis in order to determine the signaling pathway involved in $E_2$-induced KLE cell proliferation. Expression of GPR30 was confirmed in both Ishikawa and KLE cell lines, but was stronger in KLE cells (Fig. 5A). GPR30 expression and AKT phosphorylation (pAKT) were significantly increased following stimulation with 1 nM $E_2$ (Fig. 5B) in KLE cells. Moreover, GPR30 knockdown resulted in marked pAKT reduction, and $E_2$-induced phosphorylation of AKT was blocked by depletion of GPR30 (Fig 5D). Furthermore, MTT assays indicated that GPR30 knockdown resulted in decreased proliferation, and $E_2$-induced KLE cellular growth was also attenuated (Fig 5E). Further investigation of the time dependency of the $E_2$-induced PI3K/AKT signaling demonstrated that LY294002, a specific inhibitor of the PI3K/AKT signaling pathway, clearly inhibited pAKT expression and was associated with KLE cell growth arrest both in the presence and in the absence of $E_2$ (Fig. 5C and E).

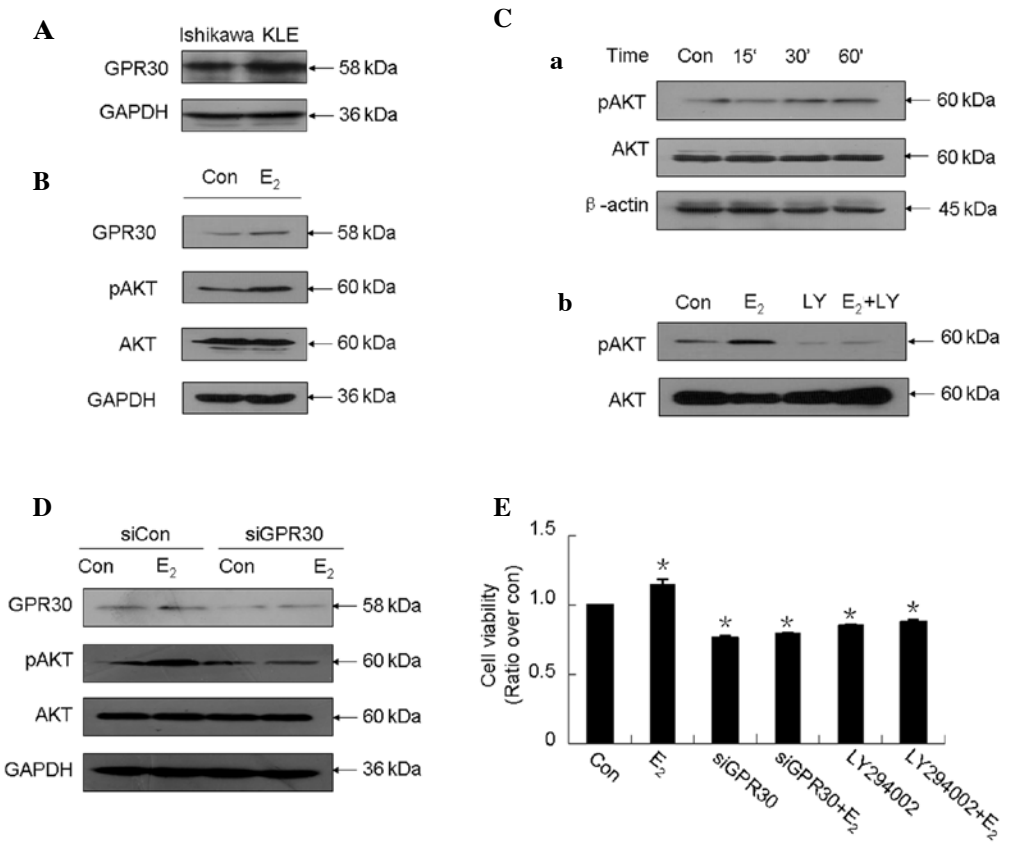


Figure 5. Estrogen-induced KLE cell expansion is GPR30-PI3K/AKT signal dependent. (A) Western blot analysis of GPR30 expression in nuclear ER-positive Ishikawa cells and nuclear ER-negative KLE cells. (B) $E_2$ (1 nM) up-regulated GPR30 and pAKT proteins. (C-a) The effect of $E_2$ (1 nM) on pAKT expression at different time points. (C-b) The effect of $E_2$-pAKT was abolished by LY294002 (10 $\mu$M). (D) GPR30 knockdown inhibited activation of PI3K/AKT signaling induced by $E_2$. (E) GPR30 knockdown in combination with LY294002 treatment induced KLE cell proliferation arrest; *p<0.05 compared with the control (Con).

## Discussion

Endometrial cancer is one of the most common gynecological cancers in the world, the majority of which are estrogen-driven lesions. Increasing evidence has confirmed that prolonged exposure to excessive estrogen is the classical etiological factor for endometrial cancer. However, the detailed molecular mechanisms underlying this type of tumorigenesis remain to be clarified.

Previous studies have indicated that estrogen enhances nuclear ER-positive endometrial cancer cell proliferation, whereas the effect of estrogen on nuclear ER-negative endometrial cancer cells is controversial (14,15). In this study, estrogen-induced proliferation was observed in both nuclear ER-positive Ishikawa cells and nuclear ER-negative KLE cells. It is generally believed that estrogen-enhanced endometrial cell growth is mediated by nuclear ER through a classic pathway that depends on gene transcription and protein synthesis. In the current study, it was observed that ICI-induced inhibition of nuclear ER expression attenuated estrogen-induced Ishikawa cell expansion. In contrast, estrogen treatment of nuclear ER-transfected KLE cells resulted in significantly greater proliferation than that of estrogen stimulated untransfected cells. This indicates that overexpression of nuclear ER in KLE cells restores estrogen-induced proliferation via the classical nuclear ER pathway. This study has further confirmed that activation of the nuclear ER pathway is required for nuclear ER-positive endometrial cancer cell growth and survival.

In order to further elucidate the mechanism by which estrogen-enhanced endometrial cancer cell proliferation is mediated by nuclear ER, the role of Notch signaling in cellular growth was investigated. Aberrant Notch1 expression is implicated in the development of various cancers, and Notch signaling blockade appears to affect the survival and proliferation of multiple types of cancer. Zang *et al* reported that Notch1 is overexpressed and highly activated in breast cancers, and further investigation confirmed that blockade of Notch signaling inhibited proliferation by both cell cycle arrest and apoptosis (16). Similarly, down-regulation of Notch1 has been shown to contribute to cell growth inhibition in pancreatic cancer cells (10). In the current investigation, estrogen was observed to up-regulate Notch1 mRNA expression in a dose-dependent manner in Ishikawa cells but not in KLE cells. DAPT-mediated Notch signaling blockade was shown to attenuate expression of Notch1 mRNA and protein in Ishikawa cells. Furthermore, the MTT assay confirmed that Ishikawa cell proliferation was inhibited by DAPT treatment and, more importantly, estrogen-induced cellular growth was abolished. These data provide support for the involvement of Notch signaling in the regulation of Ishikawa cell proliferation. To investigate the relationship between nuclear ER and Notch signaling, ICI was used to block ER expression. It was observed that ICI not only abolished estrogen-induced cellular growth but also attenuated estrogen-up-regulated Notch1 expression in Ishikawa cells. This indicates that estrogen-induced cell proliferation and activation of the Notch signaling pathway are mediated by nuclear ER in Ishikawa cells. Two other phenomena should be noted. Firstly, in Ishikawa cells, it was observed that Notch signaling was significantly inhibited by DAPT both in the presence and absence of estrogen. This is in accordance with previous reports (17-20) that Notch signaling is directly involved in regulation of cellular growth, although the observed changes in proliferation are not consistent with the variation in Notch1 protein expression. DAPT treatment resulted in a marginal inhibition of proliferation compared with a marked decrease in Notch1 protein expression. This observation suggested involvement of other signaling pathways in the regulation of cellular proliferation in Ishikawa cells. Secondly, estrogen enhanced KLE cell growth in the absence of any effect on Notch1 expression, indicating the involvement of an alternative signaling pathway in the regulation of KLE cell proliferation. This has been identified as the GPR30 signaling pathway in our study.

It has been shown that estradiol regulates MAPK through a sex-specific membrane-mediated mechanism that does not involve ER, thus demonstrating the existence of a non-classical pathway for estradiol (8,10,21,22). These 'non-genomic' effects have important functional roles in a variety of pathophysiological processes and do not depend on gene transcription or protein synthesis. Instead, these effects involve steroid-induced modulation of cytoplasmic or cell membrane-bound regulatory proteins (23,24) which allow more rapid responses to estrogen. Therefore, research has focused on GPR30 signaling. In a previous study, it was confirmed that overexpression of GPR30 more frequently occurred in endometrial tumors with deep myometrial invasion, high-grade, biologically aggressive histological subtypes, and advanced stage, and elevated GRP30 protein in tumors strongly associated with poor survival (25). In this study, GPR30 expression was found in both Ishikawa and KLE cell lines, and ER-negative KLE cells showed a stonger expression of GRP30 than ER-positive Ishikawa cells. This is consistent with the finding that GRP30 expession is inversely correlated with the ER status (25,26).

In this study, it was observed that estrogen treatment of KLE cells resulted in up-regulated GPR30 expression and induced activation of the PI3K/AKT pathway. Subsequent studies confirmed that depletion of GPR30 resulted in decreased expression of pAKT in the presence and absence of estrogen, thus indicating that activation of the estrogen-induced PI3K/AKT pathway is mediated by GPR30. The effects of growth conditions on KLE cellular growth were analyzed by MTT assays. Depletion of GPR30 resulted in inhibition of PI3K/AKT signaling and induced KLE proliferation arrest. These data demonstrate that estrogen regulated nuclear ER-negative endometrial cancer cell proliferation via the PI3K/AKT signaling pathway is mediated by the cell membrane receptor, GPR30. Furthermore, although GPR30 was also detected in Ishikawa cells, proliferation inhibition mediated by the blockade of the Notch signaling pathway was not as pronounced as the alteration of the Notch1 protein levels (Fig. 1D and E). It can be speculated that estrogen-induced cell expansion through the GPR30-PI3K/AKT signaling pathway partially abrogates the inhibitory effects of Notch signaling blockade in Ishikawa cells. Thus, nuclear ER-positive endometrial cancer cells may also be regulated by both signaling pathways.

In conclusion, these data demonstrate that both Notch and GPR30-PI3K/AKT signaling pathways are involved in regulation of Ishikawa cell proliferation and indicate the existence of the classical pathway mediated by nuclear ER and a

non-classical, rapid response pathway in nuclear ER-positive endometrial cancer cells, whereas only the latter is involved in regulation of cellular growth in nuclear ER-negative endometrial cancer cells. These findings provide evidence of different molecular mechanisms of cell proliferation in ER-negative and ER-positive endometrial cancer cells. These pathways represent targets for the development of specific inhibitors for more effective cancer therapies.

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