Notch activity mediates oestrogen-induced stromal cell invasion in endometriosis

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Abstract

Oestrogen has been reported to control the invasiveness of endometrial stromal cells in endometriosis. Notch signalling, a master regulator of cell invasion in tumours, is regulated by oestrogen in other diseases and hyperactivated in endometriotic stromal cells. Therefore, we hypothesized that an interaction between Notch signalling and oestrogen may exist in the regulation of endometrial stromal cell invasion, which is essential for the development of endometriosis. Western blot analysis of tissues showed that the expression levels of Notch components (JAG1 and NOTCH1) and Notch activity were markedly higher in ectopic endometria than in their eutopic and normal counterparts. Primary stromal cells obtained from normal endometria cultured with oestrogen presented significant increases in the expression of Notch components and Notch activity, the cytoplasmic and nuclear accumulation of NOTCH1 intracellular domain, the expression of matrix metalloproteinase 9 and vascular endothelial growth factor and cell invasiveness. Knockdown of *NOTCH1* markedly alleviated oestrogen-induced matrix metalloproteinase 9 and vascular endothelial growth factor expression and cell invasion. ICI (an oestrogen receptor α antagonist) also blocked these oestrogenic effects. Oestrogen-responsive elements were found in the promoters of *NOTCH1* and *JAG1*. A luciferase reporter analysis revealed that oestrogen regulated the expression of Notch components via oestrogen receptor alpha, which is bound to oestrogen-responsive elements in the *JAG1* and *NOTCH1* promoters. Collectively, our findings indicate that oestrogen engages in crosstalk with Notch signalling to regulate cell invasion in endometriosis via the activation of oestrogen receptor alpha and the enhancement of Notch activity. Notch signalling blockade may therefore be a novel therapeutic target for endometriosis.

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Introduction

Endometriosis (EMS) is a benign but metastatic disease that is characterized by the appearance of endometrial tissue outside of the uterine cavity and causes cyclic pelvic pain and infertility (Giudice & Kao 2004). The most well-accepted hypothesis for the pathogenesis of EMS is the retrograde menstruation/transplantation theory (Sampson 1927). According to this theory, the successful establishment of EMS results from a series of complicated processes, including the adherence, invasion and growth of menstrual endometrial cells which originate from retrograde endometrial fragments. Of these processes, the initial step is the invasion of endometrial stromal cells into the peritoneum is a vital step for EMS formation: a process which is similar to that observed in metastatic neoplasms (Witz *et al.* 1999). Matrix metalloproteinase 9 (MMP9) and vascular endothelial growth factor (VEGF) are genes with well-known roles in cell invasion in metastatic tumours. MMP9 enables invasion mainly by catalysing the degradation of the extracellular matrix (ECM) (Emonard & Grimaud 1990), while VEGF facilitates invasion by

interacting with VEGF receptors (VEGFRs) expressed by tumour cells in paracrine or autocrine manner (Lichtenberger *et al.* 2010). Abundant evidence has shown that MMP9 and VEGF are overexpressed in the ectopic endometrium (Di Carlo *et al.* 2009, Machado *et al.* 2010) and their concentrations in peritoneal fluids are elevated in patients with EMS (Bourlev *et al.* 2006, Liu *et al.* 2015); these changes may contribute to the invasion of endometrial cells. However, the upstream pathways that regulate the production of these two factors remain to be further investigated.

Oestrogen plays a key role in the pathogenesis of EMS (Kitawaki *et al.* 2002, Ferrero *et al.* 2014, da Costa e Silva Rde *et al.* 2016). It controls not only the maintenance and growth of endometriotic implants (Yland *et al.* 2018) but also the invasion of endometrial stromal cells. *In vitro*, oestrogen was found to indirectly regulate MMP9 expression and activity by activating Wnt/ β -catenin signalling (Xiong *et al.* 2015, Zhang *et al.* 2016a) or inducing c-Fos (a proto-oncogene) (Pan *et al.* 2016), thereby contributing to the invasion of endometrial stromal cells. Since Wnt/ β -catenin signalling and c-Fos are both essential for tumour invasion (Dong *et al.* 2015,

Paw *et al.* 2015) and EMS exhibits features of malignant tumours, we hypothesized that additional signalling pathways known to be associated with tumour invasion may also mediate oestrogen-induced invasiveness in endometrial stromal cells.

Interestingly, Notch signalling was recently found to be activated in endometriotic stromal cells (Gonzalez-Foruria *et al.* 2017, Brown *et al.* 2018). Notch signalling regulates the proliferation, apoptosis, migration, invasion and metastasis of tumour cells (Capaccione & Pine 2013). It is classically activated by the binding of receptors to ligands on adjacent cells, which leads to the cleavage of the transmembrane domain and the subsequent generation of a Notch intracellular domain (NICD) (Pancewicz & Nicot 2011, Bray 2016). NICD then translocates into the nucleus to transcriptionally activate its target genes. Overexpression of Notch signalling components and overactivation of Notch are often correlated with increased cancer cell invasiveness (Hafeez *et al.* 2009, Capaccione & Pine 2013). Knockdown of *NOTCH1* decreased the expression of extracellular proteins involved in cell invasion, including MMP9 and VEGF (Wang *et al.* 2006, Zhou *et al.* 2012). More importantly, Notch signalling has also been shown to be modulated by oestrogen in the regulation of angiogenesis, cell differentiation and proliferation (Soares *et al.* 2004, Wei *et al.* 2012, Fan *et al.* 2014). Therefore, the aim of the present study was to determine whether Notch signalling mediates oestrogen-induced invasiveness in human endometrial stromal cells in EMS and investigate the underlying molecular mechanism.

Materials and methods

Tissue collection and cell culture

Informed consent was obtained from all patients, and the study was approved by the Institutional Review Board and Research Ethics Committee of Tongji Medical School, Huazhong University of Science and Technology. As previously described (Zhang *et al.* 2016b), normal endometrial samples were obtained from 10 patients aged 27–40 years old (mean age: 33.10 years old) with tubal infertility and without endometriosis, as confirmed by laparoscopy. Paired eutopic and ectopic endometria were collected from 10 patients aged 25–44 years (mean age: 30.90 years old) with ovarian endometriosis that was confirmed histologically. According to the criteria of the revised American Society of Reproductive Medicine (R-ASRM) score classification system, all these cases were classified as stage III–IV, and samples were taken during the proliferative phase of the cycle based on the data of the last menstrual period and histology. Endometria in this phase were used to determine whether there are differences in Notch activity and components among the three kinds of endometria due to different local oestrogen concentrations (Huhtinen *et al.* 2012) while simultaneously avoiding any interference by progesterone. All patients had regular menstrual cycles and received no hormones or GnRH agonist therapy for at

least 3 months before surgery. Patients with hyperplasia of the endometrium, pelvic inflammatory diseases or malignant tumours, adenomyosis, dysfunctional uterine bleeding or systemic pathologies were excluded. Both normal and eutopic endometrial samples were only used for protein extraction, while each ectopic endometrial sample was divided into two parts, one of which was used for protein extraction, while the other was used for immunohistochemical studies.

Primary endometrial stromal cells (ESCs) obtained from normal endometria were chosen for the drug treatment experiments. As previously described (Zhang *et al.* 2016a), primary ESCs were isolated from each of the five specimens that were obtained from another set of 220 patients aged 25–43 years old (mean age: 31.93 years old) who were without endometriosis and underwent hysteroscopic curettage during the late secretory phase based on previous studies (Yoshida *et al.* 2004, Li *et al.* 2018). Endometria in this phase were used as a model for the retrograde endometrial fragments from which endometriotic stromal cells are thought to originate. We chose endometria in this phase because they were readily available and best mimicked the menstrual endometria (i.e. had the most similar tissue composition).

An established human endometrial stromal cell line (ThESC, ATCC, USA) was used in this study. Both ESCs and ThESCs were cultured in DMEM/Ham's F-12 containing 10% foetal bovine serum (FBS) in a humidified incubator containing 5% CO₂ at 37°C. The medium was changed every other day. Before steroid treatment, ESCs and ThESCs were starved for 24 h with phenol red-free and serum-free DMEM/Ham's F-12 in order to remove endogenous steroids. Then, the medium was changed again to serum-free DMEM/Ham's F-12 at the time of steroid administration.

Reagents

The 17 β -oestradiol (E2, Sigma-Aldrich) was dissolved in DMSO, stored at 80°C and diluted in PBS before use. ICI-182780 (ICI, Cayman Chemicals) was dissolved in ethanol, stored at 80°C and diluted in PBS before use.

In vitro treatments with E2 or ICI

As simultaneous administration of E2 and ICI would probably interfere with the patterns of genes expressed upon ICI treatment, cells were incubated in serum-free medium in the presence of 10⁻⁵ mol/L ICI during starvation. After 24 h of serum starvation, the ESCs were maintained in serum-free medium with E2 at 10⁻⁸ mol/L. The concentrations of E2 and ICI used in this study were based on previous studies (Wei *et al.* 2012, Zhang *et al.* 2016a). PBS + DMSO and PBS + ethanol were used as the vehicle control treatments for E2 and ICI, respectively. Each experiment was carried out in triplicate and repeated at least three times.

Western blot analysis

Equal amounts of proteins extracted from tissue samples (30 μ g) and cell samples (90 μ g) were loaded and subjected to 8% SDS-PAGE, and the following steps were performed as

previously described (Zhang *et al.* 2016a). The primary antibodies used in these experiments were JAG1 (ab85763, 1.25 µg/mL, Abcam), NOTCH1 (ab65297, 1.6 µg/mL, Abcam), VEGF (19003-1-AP, 0.73 µg/mL, Proteintech), MMP9 (10375-2-AP, 0.55 µg/mL, Proteintech), N1ICD (AF5307, 1 µg/mL, Affinity, USA) and GAPDH (AF7021, 1 µg/mL, Affinity). Optical densities (ODs) were measured by ImageJ (Rawak Software, Inc. Germany). Expression was calculated as the ratio of the target OD to the GAPDH OD in each case.

Immunohistochemical staining

Formalin-fixed paraffin-embedded tissues were sectioned at 4 µm thickness using routine deparaffinization and rehydration procedures. The sections were stained as previously described (Zhang *et al.* 2016a). The primary antibodies used in these experiments were JAG1 (#70109, 0.5 µg/mL, CST, USA), NOTCH1 (#3608, 4.2 µg/mL, CST) and N1ICD (AF5307, 5 µg/mL, Affinity). Sections incubated without the primary antibody served as negative controls, while sections obtained from squamous cell lung cancer, cervical squamous carcinoma and prostatic carcinoma that positively expressed JAG1, NOTCH1 and N1ICD respectively were used as positive controls as shown in Supplementary Fig. 1 (see section on [supplementary data](#) given at the end of this article).

Immunofluorescence

Primary stromal cells were seeded on glass coverslips placed in six-well plates. After the cells were treated with E2 for 24 h, they were fixed with 4% paraformaldehyde for 15 min, permeabilized in 0.05% Triton X-100 (Sigma) for 30 min and blocked with 5% BSA containing 0.01% Triton X-100 for 1 h at room temperature. Then, the cells were incubated with anti-N1ICD antibodies (10 µg/mL) at 4°C overnight. The next day, the cells were washed and incubated with CY3-conjugated secondary antibodies (GB21303, 1:50, Google Biological Technology, China) for 1 h at 37°C in the dark. Nuclear counterstaining was applied with DAPI (Sigma) for 5 min at 37°C, and the cells were then washed, mounted with fluorescence-quenching inhibitor and examined to determine the N1ICD distribution under an Olympus FV1000 laser-scanning confocal microscope (Olympus).

Immunocytochemistry

After 24 h of treatment with E2, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.1% Triton X-100 for 10 min. Endogenous peroxidases were blocked with 3% H₂O₂. After the cells were blocked with 10% goat serum, they were incubated with anti-N1ICD antibodies (10 µg/mL) at 4°C overnight. Then, the cells were incubated with a secondary antibody at 37°C for 30 min, stained with 3,3'-diaminobenzidine (DAB) for 5 min and counterstained with alcohol-free haematoxylin. After the cells were washed with PBS, they were visualized by light microscopy to determine the distribution of N1ICD labelling.

Migration and invasion assays

Transwell units (24-well with 8.0-mm pore-size polycarbonate filters; Corning Costar, Tewksbury, MA, USA) were used for migration and invasion assays. The approach used for the invasion assay was identical to that used for the migration assay, except that the chambers were precoated with 100 µL of 1:3 diluted Matrigel (Sigma-Aldrich) and pre-hydrated in serum-free medium. After the cells (ESCs or ThESCs) were detached in 10% trypsin and counted, they were diluted to 1 × 10⁵/mL in serum-free medium. A total of 200 µL of cells were plated in each upper chamber with or without E2 while 500 µL of complete medium containing 10% FBS was added to each lower chamber. All assays were performed at 37°C in humidified air with 5% CO₂ and 95% O₂. After 24 h (migration assay) or 48 h (invasion assay) of incubation, the cells (together with Matrigel for the invasion assay) on the upper surface of the membrane were removed, and the migrated or invaded cells on the underside of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. Then, the number of these cells was counted in 5 randomly selected fields per well at 200× magnification by light microscopy (Olympus), and the counts were averaged.

In the ICI group, ESCs were pre-treated as previously described. Similarly, to confirm that Notch signalling mediated oestrogen-induced cell invasion, ThESCs were transfected with control shRNA or *NOTCH1* shRNA 24 h before the migration and invasion assays. The transfection method is described below.

NOTCH1 knockdown and plasmid transfection

The *NOTCH1* shRNA and control shRNA were purchased from GeneChem (China). Four pairs of oligonucleotides encoding shRNAs for *NOTCH1* were designed (data not shown), and knockdown efficiency was examined by Western blot analysis. The shRNA with the best knockdown efficiency was used for subsequent experiments. The following were the most effective sequences selected in the pilot experiment: (*NOTCH1* shRNA) 5'-CCGGGCCGAACCAATACAACCCTCTCTCGAGAGAGGGTTGTATTGTTCCGGCTTTTG-3' (sense) and 5'-AATTCA A A A G C C G A A C C A A T A C A A C C C T C T C T C G A G A G A G G T T G T A T T G G T T C G G C -3' (antisense).

For plasmid transfection, 1.3 × 10⁶ ThESCs were seeded in each well of a six-well plate. At 40–60% confluence, the cells were transiently transfected with the *NOTCH1* shRNA construct or shRNA control vector using Neofect (Neofect Beijing Biotech Co. Ltd, China) according to the manufacturer's instructions. Transfection efficiency was tracked by detecting the green fluorescent protein (GFP) co-expressed by the GV248 vector. The cells were cultured for 24 h after transfection and subsequently incubated with or without E2 for another 24 h in serum-free medium before collection. Each experiment was carried out in triplicate and repeated three times.

RNA extraction and real-time PCR

Total RNA extraction, cDNA synthesis and quantitative real-time PCR were performed as previously described

(Xiong *et al.* 2016). The primer sequences are presented in Supplementary Table 1. The PCR amplification products were analysed by melting curve and 1.5% agarose gel electrophoresis, followed by sequencing to verify their authenticity. *ACTB* and *GAPDH* were used as internal controls and the data were normalized to the geometric mean of *ACTB* and *GAPDH*. Quantitation of target genes was performed using the $2^{-\Delta\Delta t}$ method. All real-time PCRs were performed in triplicate and repeated at least three times.

Luciferase reporter assay

We searched for oestrogen-responsive elements (EREs) using the JASPAR Database in the 3000 bp regions located in the 5'-flanking regions of the *JAG1* and *NOTCH1* genes. One ERE and three EREs were found in the *JAG1* and *NOTCH1* genes, respectively: the *JAG1* gene contained GCCCAGGGTGAGCACGCCCT, and the *NOTCH1* gene contained GGCCAATGTCAGGGCCT (N1a), TCCTTAGCTCACCTGACAA (N1b) and AGGCACGGGCACCCTGTGCC (N1c). The following series of constructs was generated: the wild-type *JAG1* (FLJ1) and *NOTCH1* promoters (FLN1), an ERE mutation identified in the *JAG1* promoter (MuJ1) and the ERE deletions identified in the *NOTCH1* promoter (N1a, N1b, N1c). They were cloned into the pGL3 luciferase reporter plasmid.

The ESCs were plated in 24-well plates 1 day before transfection. At 40–60% confluence, the cells were transfected with the indicated luciferase reporter constructs or the empty vector, as previously described. A pRL-SV40 (Promega) control vector containing the Renilla luciferase gene was also co-transfected at the same time for normalization of luciferase activity. Twenty-four hours after transfection, E2 was added. After another 24 h, cells were collected for the luciferase assay, which was performed using a Dual-Luciferase Assay Kit (GN201-01, YHP-Bio, China), and measurements were made

on a GloMax 20/20 luminometer (Promega). These experiments were performed in triplicate and repeated three times.

Statistical analysis

The trial number is indicated in the figure legends. All statistical analyses were performed using GraphPad InStat 7.0 (GraphPad Software, Inc). The unpaired Student's *t* test was used to identify significant differences between two groups, and one-way ANOVA with Tukey's *post hoc* analysis was used for comparisons between three or more groups. All data sets are shown as the mean $\pm$ standard deviation (S.D.) of three independent experiments. A *P* value <0.05 was considered statistically significant.

Results

Notch components and activity are increased in EMS

JAG1 and NOTCH1 are the ligand and receptor, respectively, of the Notch family. Their binding leads to the activation of Notch signalling, which can be quantified according to N1ICD levels (Bray 2006, Pancewicz & Nicot 2011). Therefore, to assess the role of Notch signalling in EMS, the protein levels of JAG1, NOTCH1 and N1ICD were analysed using Western blot in normal (control), eutopic (Eu) and ectopic (Ec) endometria. The results showed that the expression levels of JAG1 and NOTCH1 and the level of N1ICD were significantly higher in the Ec and Eu groups than in the control group ($n=10$, Fig. 1). Additionally, all three were present at higher levels in the Ec group than in the Eu group ($n=10$, Fig. 1). These results suggest that Notch signalling is hyperactivated in EMS and might therefore be associated with the development of EMS.

Figure 1 JAG1, NOTCH1 and NOTCH1 intracellular domain (N1ICD) levels are significantly higher in eutopic and ectopic endometria in endometriosis than in normal endometria. (A) Left: Western blot analysis of JAG1, NOTCH1 and N1ICD protein levels in normal endometria obtained from patients ($n=10$) with tubal infertility and paired eutopic and ectopic endometria obtained from patients ($n=10$) with endometriosis. GAPDH was used as the internal control. Right: Bars indicate relative expression levels after normalization to GAPDH. Mean values were obtained from $n=3$ independent experiments; $*P<0.05$ by one-way ANOVA. Normal, normal endometria; Eu, eutopic endometria; Ec, ectopic endometria; N1ICD, NOTCH1 intracellular domain. (B) Representative photomicrograph of immunohistochemical staining for the JAG1 (left), NOTCH1 (middle) and N1ICD (right) proteins in human endometriotic endometrium.

To further examine the cell-specific expression of Notch components and distribution of N1ICD in EMS, we next performed an immunohistochemical analysis. Representative images of immunostaining for JAG1, NOTCH1 and N1ICD are shown in Fig. 1B. Both NOTCH1 and JAG1 were predominantly expressed in epithelial cells and weakly expressed in stromal cells in endometriotic lesions. In contrast, N1ICD was highly expressed in both epithelial cells and stromal cells. For intracellular localization, both JAG1 and NOTCH1 were localized at the cell membrane, while N1ICD was localized in cell plasma and predominantly in cell nucleus. These results suggest that Notch activation may be associated with the role of stromal cells in EMS.

Oestrogen enhances Notch signalling and the invasiveness of ESCs

Since oestrogen concentration is elevated in endometriotic lesions (Bulun *et al.* 2000) and oestrogen plays a role in regulating Notch signalling in other diseases, we next examined the changes in Notch components and activity in response to exogenous E2 in normal primary ESCs. After treatment with E2, the protein levels of JAG1, NOTCH1 and N1ICD were all strongly upregulated in a time-dependent manner (Fig. 2A), indicating that increased levels of Notch components and activity in endometriotic lesions maybe associated with the local high concentration of E2. Together with

Figure 2 Oestrogen increases Notch activity (assessed as the N1ICD level), the protein levels of JAG1, NOTCH1, MMP9 and VEGF and the intracellular distribution of N1ICD. (A) The protein levels of JAG1, NOTCH1, N1ICD, MMP9 and VEGF by time course analysis after treatment with 17 β -oestradiol (E2) as determined by Western blot. The bars indicate relative expression levels after normalization to GAPDH. Mean values were obtained from $n=3$ independent experiments; * $P<0.05$ by one-way ANOVA. n.s., not significant. (B) Immunofluorescence showing the subcellular localization of N1ICD in the control (PBS + DMSO-treated) and E2-treated groups. (C) Immunohistochemistry showing the subcellular localization of N1ICD in the control and E2-treated groups. The scale bars represent 10, 5 and 2.5 μ m, as indicated.

the previous observation that oestrogen promoted JAG1-induced Notch signalling activity (Soares *et al.* 2004), our findings indicate that E2 may enhance Notch activity by increasing the levels of Notch components and NOTCH1 cleavage.

To further examine the effect of oestrogen on Notch activity, we next performed immunofluorescence and immunochemistry to morphologically define the cellular distribution of N1ICD. The immunofluorescence results showed that N1ICD was detected in a punctate pattern that was distributed in the cytoplasm and nucleus (Fig. 2B). In addition, exposure to E2 induced a striking increase in the signal intensity in both locations, especially in the nucleus. Immunochemical analysis revealed a similar pattern, and within the cytoplasm, the increase in the accumulation of N1ICD was observed mainly around the nucleus (Fig. 2C), suggesting an increase in the translocation of N1ICD into the nucleus after E2 treatment.

To explore the role of oestrogen-induced Notch signalling activation in the regulation of ESC invasiveness, we simultaneously examined MMP9 and VEGF expression and cell invasiveness following exposure to oestrogen. Consistent with our previous findings (Zhang *et al.* 2016a,b), Western blot analysis showed that the protein levels of MMP9 and VEGF were significantly higher after E2 treatment (Fig. 2A). Migration and invasion assays also showed that more cells migrated and invaded when they were incubated with E2 (Fig. 3). These data suggest that oestrogen-induced Notch activity may be correlated with oestrogen-induced ESC invasiveness.

Knockdown of NOTCH1 alleviates the E2-mediated migration and invasion of ThESCs

To confirm our hypothesis that Notch signalling may mediate the effects of oestrogen on the migration and invasion of ESCs, shRNA-mediated RNA interference was utilized to suppress NOTCH1 expression in ThESCs. ThESCs were pre-transfected with NOTCH1 shRNA or

control shRNA before treatment with E2. The results of Western blot analysis showed that knockdown of NOTCH1 significantly lowered the E2-induced expression of MMP9 and VEGF proteins (Fig. 4A) and resulted in low-level migration and invasion in cells incubated with E2 (Fig. 4B and C). This observation suggests that Notch activity is indeed involved in the regulation of E2-induced ThESC invasiveness. Notably, even in the absence of E2, NOTCH1 knockout still reduced MMP9 and VEGF expression and cell invasiveness, consistent with the results reported in the literature (Wang *et al.* 2006, Zhou *et al.* 2012) and implying that Notch signalling itself has a controlling effect on endometrial cell invasion in the absence of E2.

Oestrogen-induced Notch activity and cell invasiveness are mediated by oestrogen receptor alpha (ER α)

Oestrogen activity is primarily mediated by oestrogen nuclear receptors (Dutertre & Smith 2000, Thomas & Gustafsson 2011), with ER α playing a pivotal role in the pathogenesis of EMS (Burns *et al.* 2012). Therefore, the ER α antagonist ICI was used to characterize the molecular mechanism of oestrogen in the regulation of Notch signalling and cell invasiveness. Both PCR and Western blot analyses showed that ICI inhibited the expression of JAG1 and NOTCH1 and reduced the levels of N1ICD that were induced by E2 (Fig. 5A and B). Similarly, the E2-mediated increases in MMP9 and VEGF expression and cell migration and invasion were also blocked by ICI (Fig. 5A, B and C). These data suggest that nuclear ER α is essential for E2 actions in terms of Notch signalling and endometrial stromal cell invasiveness.

E2 upregulates Notch signalling components by activating oestrogen receptor elements (EREs)

Nuclear ERs usually act as transcription factors by binding to EREs in downstream target genes to regulate their expression (Dutertre & Smith 2000, Thomas & Gustafsson 2011). In this study, RT-PCR analysis

Figure 3 E2 enhances the migratory capacity and invasiveness of endometrial stromal cells (ESCs). Left: Representative photographs of ESC migration and invasion in the control (PBS + DMSO-treated) and E2-treated groups. Original magnification, 200 $\times$. Right: Quantified results of the migration and invasion assays. Mean values were obtained from $n=3$ independent experiments; * $P<0.05$ by unpaired Student's t -test.

Figure 4 Knockdown of *NOTCH1* by shRNA transfection alleviates oestrogen-mediated MMP9, VEGF expression and ThESC migration and invasion. (A) ThESCs were pre-transfected with scramble shRNA or *NOTCH1* shRNA. *NOTCH1* knockdown inhibited E2-induced MMP9 and VEGF expression determined by Western blot analysis. Bars indicate relative expression levels after normalization to GAPDH. Mean values were obtained from $n=3$ independent experiments; $*P<0.05$ by one-way ANOVA. (B) Representative photographs of ThESC migration and invasion after transfection with scrambled shRNA or *NOTCH1* shRNA in the presence or absence of E2. (C) Bars correspond to the quantified results of the migration and invasion assays. Mean values were obtained from $n=3$ independent experiments; $*P<0.05$ by one-way ANOVA.

indicated that the effect of E2 on Notch components was mediated by transcriptional regulation (Fig. 5A). Whether oestrogen activates the transcription of Notch signalling components by binding to EREs is unknown. Therefore, to further investigate the potential molecular link between E2 and Notch signalling, luciferase assays were conducted. One and three EREs were found in the 5'-flanking regions of the *JAG1* and *NOTCH1* genes, respectively (Fig. 6A). E2 stimulated an increase in luciferase activity from FLJ1 (full-length *JAG1* promoter from -2970 to -2951bp), which contains a putative ERE, but not from MuJ1 (full-length *JAG1* promoter with mutated ERE) (Fig. 6B), suggesting that the region from -2970 to -2951 containing the putative ERE in the *JAG1* promoter is oestrogen responsive. Additionally, E2 also induced luciferase activity from the FLN1 construct (full-length *NOTCH1* promoter from -3000 to 0bp) (Fig. 6A and C), which contains three putative EREs (from -70 to -51, -89 to -70, and -1870 to -1851bp). When any of the three putative EREs was deleted, luciferase activity was no longer stimulated by E2, indicating that all three putative EREs in the *NOTCH1* promoter were needed for oestrogen responsiveness. Altogether, these results indicate that E2 upregulates the *NOTCH1* and *JAG1* genes via nuclear ER α interactions with *JAG1* and *NOTCH1* promoter regions that contain putative EREs.

Discussion

Oestrogen and Notch signalling engage in crosstalk to regulate a wide range of biological functions, such as tumour angiogenesis (Soares *et al.* 2004) and neuronal development (Ruiz-Palmero *et al.* 2011). However, whether the two signals are correlated in terms of cell invasiveness is unknown. Notch signalling has been recently demonstrated to be associated with fibrosis and angiogenesis in EMS. Whether it has a relationship with the invasion and metastasis of EMS is also unknown. In the current study, we found that Notch signalling was involved in the oestrogen-induced migration and invasion of ESCs, and these processes might facilitate the establishment and metastasis of EMS. In normal ESCs, oestrogen activates Notch signalling, upregulates its components (*JAG1* and *NOTCH1*) in an ER α -dependent manner and transcriptionally regulates *JAG1* and *NOTCH1* expression by binding EREs in the promoters of these two genes. Notch signalling additionally mediates oestrogen-induced cell invasion by modulating the expression of MMP9 and VEGF.

JAG1 and *NOTCH1* overexpression and high Notch activity are usually associated with increased cell invasiveness (Mitsuhashi *et al.* 2012, Bednars-Knoll *et al.* 2016). In EMS, the migration and invasion capacities

Figure 5 ER α mediates the oestrogen-induced activation of Notch signalling, the upregulation of JAG1, NOTCH1, MMP9 and VEGF, and the enhancement of migration and invasion of ESCs. (A and B) JAG1, NOTCH1, MMP9 and VEGF mRNA (A) and protein (B) levels together with N1ICD protein levels (B) were analysed after E2 and ICI treatment by real time-PCR (A) and Western blot (B) analysis. Bars indicate relative expression levels after normalization to GAPDH. Mean values were obtained from $n=3$ independent experiments; * $P<0.05$ by one-way ANOVA. (C) Representative photographs of migration and invasion of ESCs treated with or without ICI (left). Bars show the quantified results of migration and invasion assays (right). Mean values were obtained from $n=3$ independent experiments; * $P<0.05$ by one-way ANOVA.

of ectopic ESCs are stronger than those of eutopic and normal ESCs (Guan *et al.* 2016). Our findings that JAG1 and NOTCH1 expression and Notch activity were increased in endometriotic lesions (Fig. 1A) and that N1ICD was highly expressed in ectopic ESCs (Fig. 1B) suggest that Notch signalling may be associated with the enhanced invasiveness of ectopic ESCs. Moreover, our study provides laboratory evidence showing that oestrogen can upregulate the expression of JAG1, NOTCH1 and activate Notch signalling. Therefore, these data imply that the hyperactivation of Notch signalling in ectopic ESCs may be attributed to the locally high oestrogen concentrations observed in EMS (Huhtinen *et al.* 2012). Additionally, Notch activation may result from the combined effects of the upregulation of NOTCH1 and JAG1 expression and NOTCH1 cleavage since the binding of NOTCH1 to JAG1 leads to the activation of Notch signalling and NOTCH1 cleavage.

The role of oestrogen in the regulation of Notch signalling and cell invasion depends on its receptors. The predominant oestrogen receptors in endometriotic lesions are ER α and ER β . There is some controversy in the literature regarding the role of ER α in the regulation of Notch signalling. Soares and his colleagues found that oestrogen activated Notch signalling through ER α (Soares *et al.* 2004), while Rizzo *et al.* found that oestrogen inhibited Notch signalling via ER α by blocking the cleavage of NOTCH1 (Rizzo *et al.* 2008). Our data support the report by Soares. We are the first to provide evidence showing that ER α plays a facilitative role in modulating the invasiveness of ESCs by activating Notch signalling. We also directly measured N1ICD protein levels instead of NOTCH1 and thereby avoided the measurement limitations associated with reporter assays or antibodies for NOTCH1 in Soares' and Rizzol's

studies, respectively. In those reports, it is possible that Notch activation might have been masked by increased transfection by E2 in reporter assays (Soares *et al.* 2004) or that the NOTCH1 antibody might not have recognized NOTCH1 after cleavage (Rizzo *et al.* 2008). Nevertheless, one cannot ignore that many studies have demonstrated that ER α levels are decreased while ER β levels are dramatically increased in ectopic lesions and ESCs in EMS (Xue *et al.* 2007, Bulun *et al.* 2012) in spite of our confirmation of the positive role of ER α in cell invasion. Compared to normal endometrium, in eutopic endometrium, ER α levels were also found to be higher or unchanged (Prentke *et al.* 1992, Cavallini *et al.* 2011, Pellegrini *et al.* 2012, Zhang *et al.* 2016b). Although ER β frequently exerts a suppressive effect on cell invasion in tumours (Thomas & Gustafsson 2011), it was recently confirmed to promote the invasion of epithelial cells via the involvement in TNF- α induced epithelial mesenchymal transition (EMT) in EMS. Therefore, we speculated that the upregulation of ER β in EMS may exert compensatory effects for ER α decrease to promote cell invasion, which leads to more invasive ectopic ESCs. Further investigations into the effect of ER β on Notch signalling and cell invasion in EMS are needed in the future.

Nuclear ERs classically act as transcription factors by binding to the EREs of target genes to regulate their expression. Our study confirmed that oestrogen could activate NOTCH1 and JAG1 transcription by binding to the EREs in their promoters. This result was consistent with Soares' observation for JAG1 (Soares *et al.* 2004). However, in our study, we found three EREs for NOTCH1 that worked cooperatively towards transcriptional activation, while no functional EREs were found in Soares' study. The difference in these results

Figure 6 The role of oestrogen in the activation of EREs in the promoters of *JAG1* and *NOTCH1* was assessed by luciferase assays. (A) Constructs: Full-length *JAG1* promoter containing the putative ERE (FLJ1) and mutated ERE (MuJ1), full-length *NOTCH1* promoter (FLN1) containing three putative EREs, and FLN1 with EREs deletion (N1a, N1b and N1c, respectively). (B and C) Luciferase activity after transfection of different constructs. Mean values were obtained from $n=3$ independent experiments; $*P<0.05$ by one-way ANOVA.

may be due to differences in the EREs that were found or differences in cell context. Moreover, oestrogen had a stronger pro-transcription effect on *JAG1* than on *NOTCH1* (Figs 5A, 6B and C), even though more EREs were found in the *NOTCH1* gene, indicating that other regulatory mechanism may be involved in the ER α -mediated regulation of *JAG1*. For example, nuclear ERs can regulate target genes by recruiting coactivators that bind to the promoters of target genes in the absence of ERE binding (Bjornstrom & Sjoberg 2005).

MMP9 and VEGF are well-known metastasis-related genes that regulate cell invasion by promoting the degradation of ECM and interacting with receptors expressed by tumour cells respectively. Moreover, the fragments resulted from the MMP9-mediated cleavage of ECM can also regulate cell invasion directly or affect other cellular signalling mechanisms that modulate cell invasion (Egeblad & Werb 2002). Both MMP9 and VEGF are downstream effectors of Notch signalling in the regulation of tumour invasion (Wang *et al.* 2006, Hafeez *et al.* 2009). *NOTCH1* might directly regulate the expression of MMP9 by enhancing its promoter activity or affect MMP9 level via a posttranslational stabilization mechanism (Hafeez *et al.* 2009). Our group previously confirmed that MMP9 and VEGF are involved in the oestrogen-induced invasiveness of ESCs in EMS (Xiong *et al.* 2015). The results of this study are consistent with our previous results. Moreover, knockdown of *NOTCH1* abrogated oestrogen-induced cell invasion and upregulation of MMP9 and VEGF, indicating that oestrogen regulates the invasion of ESCs via Notch signalling and the expression of MMP9 and VEGF. Notably, Notch signalling alone can regulate the

invasiveness of ESCs via MMP9 and VEGF. Thus, we provide evidence showing that oestrogen enhances the invasive capacity of ESCs by activating Notch signalling to upregulate the expression of MMP9 and VEGF, thereby leading to increased invasiveness in endometrial cells.

Our finding that Notch activity mediated oestrogen-induced cell invasion in EMS also has clinical implications. It is well known that medical treatments based on oestrogen depletion are usually associated with high recurrence rates and unexpected side effects. Combinatorial treatment, namely oestrogen depletion concomitant with Notch blockage, may produce synergistic effects and efficiently inhibit the development and metastasis of EMS. It may also be possible that Notch inhibitors or other knockdown techniques are, on their own, alternative nonhormonal treatments for EMS. Moreover, the expression levels of key Notch molecules are higher in ectopic than in normal endometria, indicating that Notch components could be utilized as biomarkers for the diagnosis of EMS.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0326>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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