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Notch-1 Signaling Activation and Progesterone Receptor Expression in Ectopic Lesions of Women With Endometriosis

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Context: Progesterone (P) resistance is a hallmark of endometriosis, but the underlying mechanism(s) for loss of P sensitivity leading to lesion establishment remains poorly understood.

Objective: To evaluate the association between Notch-1 signaling activation and P resistance in the progression of endometriosis.

Design: Case control study; archived formalin-fixed, paraffin-embedded tissues.

Setting: University hospitals (United States, Taiwan).

Patients: Women with endometriosis; human endometrial stromal cell line (HESC).

Intervention: Eutopic endometria (EU) and ectopic lesions (ECs) were collected from surgically diagnosed patients. Archived tissue sections of EU and ECs were identified. HESCs were treated with *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-S-phenylglycine *t*-butyl ester (DAPT) and valproic acid (VPA) to, respectively, suppress and induce Notch-1 activation.

Outcome Measures: Tissues were analyzed for Notch Intra-Cellular Domain 1 (NICD1) and progesterone receptor (PGR) protein expression by immunohistochemistry and for transcript levels of NICD1 target genes *HES1*, *PGR*, and *PGR-B* by quantitative reverse transcription polymerase chain reaction. DAPT- or VPA-treated HESCs with and without P cotreatment were evaluated for cell numbers and for *PGR*, *HES1*, and PGR target gene *DKK1* transcript levels.

Results: Nuclear-localized stromal NICD1 protein levels were inversely associated with those of total PGR in EU and ECs. Stromal ECs displayed higher *HES1* and lower total *PGR* and *PGR-B* transcript levels than EU. In HESCs, DAPT reduction of NICD1 decreased cell numbers and increased *PGR* transcript and nuclear PGR protein levels and, with P cotreatment, maintained P sensitivity. Conversely, VPA induction of NICD1 decreased *PGR* transcript levels and, with P cotreatment, abrogated P-induced *DKK1* and maintained *HES1* transcript levels.

Conclusions: Aberrant Notch-1 activation is associated with decreased PGR that contributes to P resistance in endometriosis.

Abbreviations: ATCC, American Type Culture Collection; CS-BCS, charcoal-stripped bovine calf serum; DAPI, 4′,6-diamidino-2-phenylindole; DAPT, *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-S-phenylglycine *t*-butyl ester; *DKK1*, Dickkopf-related protein 1; DMSO, dimethyl sulfoxide; EC, ectopic lesion; *ESR1*, estrogen receptor-α; *ESR2*, estrogen receptor-β; EU, eutopic endometria; *HES1*, Hairy and Enhancer of Split-1; HESC, human endometrial stromal cell line; *JAG2*, Jagged 2; NICD1, Notch Intra-Cellular Domain 1; P, progesterone; PGR, progesterone receptor; RRID, Research Resource Identifier; SD, standard deviation; UAMS, University of Arkansas for Medical Sciences; VPA, valproic acid.

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Endometriosis is an estrogen-dependent inflammatory disorder defined by growth of endometrial fragments outside of the uterine cavity. With its comorbid conditions of infertility, pregnancy loss, chronic pain [1–4], and increased risks for ovarian cancer [5] and coronary heart disease [6], endometriosis affects 6% to 10% of all reproductive-aged women and has an annual economic impact of >$60 billion in the United States alone. Genomic and proteomic studies have identified many molecules that differed in expression between ectopic lesions (ECs) and eutopic endometria (EU) from diagnosed patients and with those of women without disease [7–9]. Nevertheless, the causes and mechanisms of lesion establishment remain largely undefined. Understanding the complex regulatory network underlying endometriosis is requisite for its prevention, diagnosis, and effective treatment.

Progesterone (P) resistance is a major component in the constellation of disorders that characterize endometriosis [1, 3, 10, 11]. Using an immunocompetent mouse model of endometriosis recently established by our group [12], we found that activation of Notch-1 signaling [measured by increased levels of Notch Intra-Cellular Domain 1 (NICD1)] is associated with decreased expression of progesterone receptor (PGR) in ECs. This is consistent with the report that reduced PGR expression resulted from aberrant activation of Notch-1 signaling in mouse uteri [13]. In that study, the inverse association between Notch-1 activation and PGR expression was linked to infertility, but a potential link with endometriosis was not investigated. Intriguingly, an earlier report from the same group showed that EU of women with endometriosis had decreased Notch-1 signaling that led to impaired decidualization [14], a condition associated with P resistance [15]. These findings suggest that Notch-1 function is endometrial cell context-dependent, analogous to that demonstrated in many cancer types [16, 17].

Current management of endometriosis is based on pharmacological treatments and surgical intervention [18, 19]; however, the efficacy of clinically used drugs is limited and the recurrence rate for endometriosis is ~50% within 3 years after surgery or cessation of treatment. In cancers, increased cell proliferation, survival, and inflammation and decreased steroid hormone responsiveness—all features of endometriotic lesions—are associated with Notch-1 activation [20, 21]. Mouse models of human cancers and clinical trials for hormone-dependent human cancers (*e.g.*, breast, prostate) have demonstrated that use of pharmacological inhibitors of Notch-1 signaling in conjunction with antitumor drugs can inhibit tumor growth with some success [22–24]. Because blockage of Notch-1 activation may constitute a unique intervention for clinical management of endometriosis, studies to further understand the putative link between Notch signaling and endometriosis are warranted.

To address the relationship between Notch-1 activation and P resistance in human endometriosis, we compared EU and ECs for expression levels of PGR and Notch-1 signaling effector (NICD1), ligand [Jagged 2 (*JAG2*)], and gene target [Hairy and Enhancer of Split-1 (*HES1*)] and used an immortalized, P-responsive HESC [25] treated with pharmacological modifiers of Notch-1 signaling to assess effects on PGR and PGR target [Dickkopf-related protein 1 (*DKK1*)] expression. Our data provide evidence to functionally link Notch-1 activation with diminished P sensitivity via a reduction in PGR expression.

1. Materials and Methods

A. Tissue Samples (UAMS Cohort)

Archival formalin-fixed, paraffin-embedded paired endometrial EU and ECs were identified by our team pathologist (C.M.Q.) from interrogation of Department of Obstetrics and Gynecology pathology records (2011 to 2013), following protocols approved by the Institutional Review Board of the University of Arkansas for Medical Sciences (UAMS). Sections were retrieved for women (n = 5) that met the selection criteria (premenopausal, peritoneal endometriosis). Endometriosis stage was classified based on the American Society of Reproductive Medicine guidelines [26]. Tissue sections were processed for evaluation of NICD1 and PGR expression by immunohistochemistry (see below).

B. Study Population and Tissues (Taiwan Cohort)

Endometrial tissue (EU) and EC lesions from nonpregnant women with surgically diagnosed endometriosis were obtained during laparoscopy or surgeries performed at the Department of Obstetrics and Gynecology of the National Cheng Kung University Hospital (Tainan, Taiwan), following protocols approved by the Institution's Review Board. Participants signed informed consent and were without any hormonal treatments for 6 months prior to surgery. Nonpaired EU (n = 11) and EC (n = 8) samples were obtained in the first set of tissue collection, while the second set comprised of paired EU and EC samples (n = 6). Data available for the participants included endometriosis stage following established classification [26], menstrual cycle phase at collection, age and body mass index. All lesions listed in Supplemental Table 2 were peritoneal endometriosis whereas two of the six lesions in Supplemental Table 3 were classified as endometriomas. Tissues were paraffin embedded and processed for immunostaining with anti-NICD1 antibodies (see below).

C. Immunohistochemistry

Sections (5 μm) of formalin-fixed, paraffin-embedded tissues were dewaxed with xylene, rehydrated through a graded alcohol series, and subjected to antigen retrieval in citrate buffer (10 mM, pH 6.0) with boiling for 15 minutes. Sections were treated with 3% hydrogen peroxide to quench endogenous peroxidase activity and incubated in blocking reagent (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA) for 1 hour. The antibodies [Research Resource Identifier (RRID), www.antibodyregistry.org] and their respective working dilutions (used at incubation conditions of 4°C; 16 to 24 hours) are as follows: (1) rabbit anti-human Notch 1 (RRID:AB_1977387, 1:500) [27]; (2) rabbit anti-human PGR (RRID:AB_2164331, 1:200) [28]; (3) rabbit anti-human ESR1 (RRID:AB_631470, 1:200) [29]; (4) rabbit anti-human ESR2 (RRID:AB_310195, 1:200) [30]; and (5) rabbit anti-Ki67 monoclonal (RRID:AB_302459, 1:100) [31]. Sections were subsequently washed in PBS, incubated with biotinylated anti-rabbit secondary antibodies (Vector Laboratories Inc.) for 1 hour at room temperature and after additional washings, were treated with 3,3′-diaminobenzidine tetra-hydrochloride (Dako Inc., Carpenteria, CA) followed by counterstaining with hematoxylin. Normal rabbit IgG was used as a negative control for staining. For the UAMS sample cohort, the stained sections were captured by a digital pathology whole slide scanner (Aperio Image Scope; Leica Biosystems, Buffalo Grove, IL), and the number of nuclear-stained stromal cells in three to four random visual fields for each tissue section was counted. Data are expressed as a percentage of the number of nuclear-stained cells, relative to the total number of cells counted. For the Taiwan sample cohort, NICD1-immunostained cells in EU (n = 17) and ECs (n = 14) were visualized under a light microscope and evaluated in four to five random fields per slide per tissue sample. Histoscore (H-score) was calculated by a semiquantitative assessment of staining intensities in positively stained cells on a scale of 0-3 (0 = none, weak = 1, moderate = 2, strong = 3) performed by two individuals blinded to the study.

D. Primary Stromal Cell Isolation

Primary stromal cells from paired EU and ECs were isolated and evaluated for transcript levels of Notch-1 signaling components *HES1* and *JAG2* and of total *PGR* and *PGR-B* isoform. The isolation of stromal cells and confirmation of purity (>99%) by immunostaining with vimentin (stromal cell–specific) and cytokeratin (epithelial cell–specific) antibodies followed previously described protocols [32, 33].

E. RNA Extraction and Quantitative Real-Time Polymerase Chain Reaction

Total RNA was isolated using TRIsure (Taiwan-Bioline USA Inc., Boston, MA) or TRIzol (UAMS-Invitrogen, Carlsbad, CA), following the manufacturer's instructions. Total RNA (250 ng) was reverse transcribed to cDNA (iScript cDNA Synthesis Kit; Bio-Rad Laboratories, Hercules, CA) and used for SYBR green-based real-time PCR (Taiwan: Roche Diagnostics, San Francisco, CA; UAMS: Bio-Rad Laboratories), following our previously published protocols [12, 32–34]. Intron-flanking primers were designed to eliminate genomic DNA amplification using the Primer Express software (Applied Biosystems Inc., San Francisco, CA) and were obtained from Integrated DNA Biotechnologies, Inc. (Coralville, IA). Target mRNA abundance was normalized to 18S rRNA (for *HES1*, *JAG2*, *PGR*, and *PGR-B* in EU and ECs) or TATA-Binding Protein mRNA (for *HES1*, *DKK1*, and *PGR* in HESCs).

F. Cell Culture and Treatments

The HESC (ATCC CRL-4003) was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and was authenticated by the supplier using short tandem repeat DNA profiling. Cells were propagated following procedures described by ATCC and seeded at 5×10^3 cells/well onto 96-well culture plates (Corning Ware, Woodside, NY) for cell counts or 1×10^5 cells/well onto 12-well culture plates (Corning Ware) for gene expression analysis. Cells were cultured to 50% to 70% confluence in 10% charcoal-stripped bovine calf serum (CS-BCS; Invitrogen), synchronized by serum starvation in 2% CS-BCS for 24 hours, and then treated with γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-*l*-alanyl]-S-phenylglycine *t*-butyl ester (DAPT; Sigma-Aldrich, St. Louis, MO) [35] or histone deacetylase inhibitor valproic acid (VPA; Sigma-Aldrich) [36] in 10% CS-BCS at the concentration indicated for each experiment. Vehicle control treatments were dimethyl sulfoxide (DMSO; Thermo-Fischer, Waltham, MA) for DAPT and PBS for VPA in culture media. Cells were incubated with DAPT or VPA (and corresponding vehicles) for 48 hours prior to cell count assays (Hemocytometer; Thermo-Fischer). In cotreatment studies with P (medroxyprogesterone acetate, Sigma-Aldrich), cells were treated with either DAPT or VPA for 48 hours prior to P treatment (1 μM) for an additional 24 hours. Treatments for cell count assays were performed in quadruplicate and each experiment was repeated three times. To determine the effects of DAPT and VPA alone or in combination with P on gene expression, treatments were performed in triplicate, and each experiment was repeated three times.

G. Immunocytochemistry

HESCs were grown to ~70% confluence on glass coverslips placed in six-well culture dishes and incubated in media supplemented with DAPT (10 μM) or VPA (2 mM) for 48 hours. Treated cells were then fixed in 4% paraformaldehyde for 10 minutes, permeabilized with 0.2% Triton X-100 for 30 minutes, and blocked with 1% goat serum blocking solution (Vector Laboratories Inc.) for 1 hour; all procedures were carried out at room temperature. The cells were incubated with anti-NICD1 (1:200 dilution) or anti-PGR (1:200 dilution) antibodies at 4°C overnight, washed twice with PBS, and then incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibodies (1:200 dilution; Thermo-Fischer) for 1 hour at room temperature in a dark room. Cells were treated with 4′,6-diamidino-2-phenylindole (DAPI, 0.3 μM; Vector Laboratories) to stain for nuclei. Fluorescence images were collected

using a Cytation 5 Multi-Mode Reader (BioTek Instruments; Winooski, VT). The nuclear distribution (*i.e.*, colocalized DAPI and fluorescein isothiocyanate fluorescence) of NICD1 and of PGR was evaluated from four random fields for each slide at ×200 magnification, with three slides analyzed per treatment group. Data are expressed as a percentage of the numbers of nuclear-stained cells relative to the total number of cells in a given field.

H. Statistical Analyses

Numeric data are presented as mean ± Standard Deviation (SD) and were compared between experimental groups by *t* test (for two groups) or one-way analysis of variance, followed by Tukey *post hoc* test (for three or greater groups), using SigmaStat version 3.5 software (SPSS Inc., Chicago, IL). A P value ≤ 0.05 was considered to be statistically significant.

2. Results

A. Notch-1 Activation and Expression of Endometriosis Biomarkers in ECs

To investigate the potential contribution of Notch-1 signaling to human endometriosis, we evaluated the expression levels of nuclear-localized NICD1, a measure of Notch-1 signaling activation, together with those of PGR and other biomarkers of endometriosis, in paired EU and ECs of women with endometriosis (UAMS cohort). Representative immunostaining images for NICD1 [Fig. 1(a)] and PGR [Fig. 1(b)] are shown. Relative to EU, ECs showed higher and lower percentages of stromal cells with nuclear-localized NICD1 and PGR, respectively [Fig. 1(c)]. Moreover, ECs displayed higher percentages of stromal cells expressing the proliferation marker Ki67 and estrogen receptor-β (ESR2) in nuclei, than EU. However, the percentage of nuclear estrogen receptor-α (ESR1)–expressing cells was comparable between EU and ECs [Fig. 1(d)].

B. Inverse Association of Notch-1 Signaling and PGR in ECs

To confirm the induction of Notch-1 signaling in ECs, EU and ECs from women with endometriosis were collected during surgery (Taiwan cohort) and immunostained for NICD1. Staining intensities were semiquantitatively scored using a range of 0 to 3, with highest intensity designated as 3 (strong) > 2 (moderate) > 1 (weak), with no staining indicated as 0. ECs showed higher H-scores than EU for stromal NICD1 [Fig. 2(a)], in agreement with that shown for the UAMS samples (Fig. 1).

Paired EU and ECs collected from another set of women with endometriosis (n = 6) were evaluated for an association between NICD1 activation and PGR expression. Stromal cells were isolated from each tissue sample and assayed (without further culture) for transcript levels of *HES1*, *JAG2*, total *PGR*, and *PGR-B*. Stromal *HES1* transcript levels were higher for ECs than for EU, whereas those for *JAG2* did not differ between EU and ECs [Fig. 2(b)]. Total *PGR* mRNA levels were higher for stromal EU than ECs, and this trend was mimicked by *PGR-B* [Fig. 2(c)]. However, although total *PGR* mRNA levels decreased by ~50% in ECs relative to EU, *PGR-B* mRNA levels in ECs showed a greater degree of reduction (by ~75%) when compared with EU.

C. Pharmacological Modification of Notch-1 Signaling and PGR Expression

To evaluate the direct effects of Notch-1 signaling activation on endometrial stromal cells, two well-known pharmacological modifiers of Notch-1 signaling were used to alter basal levels of activated Notch-1 in the human endometrial cell line HESC. DAPT, a γ-secretase inhibitor blocks Notch signaling by inhibiting Notch endoproteolysis to its activated form NICD1 [35]. Conversely, the histone deacetylase inhibitor VPA activates the Notch signaling cascade, leading to increased levels of NICD1 [36]. Treatment of HESCs with DAPT or VPA

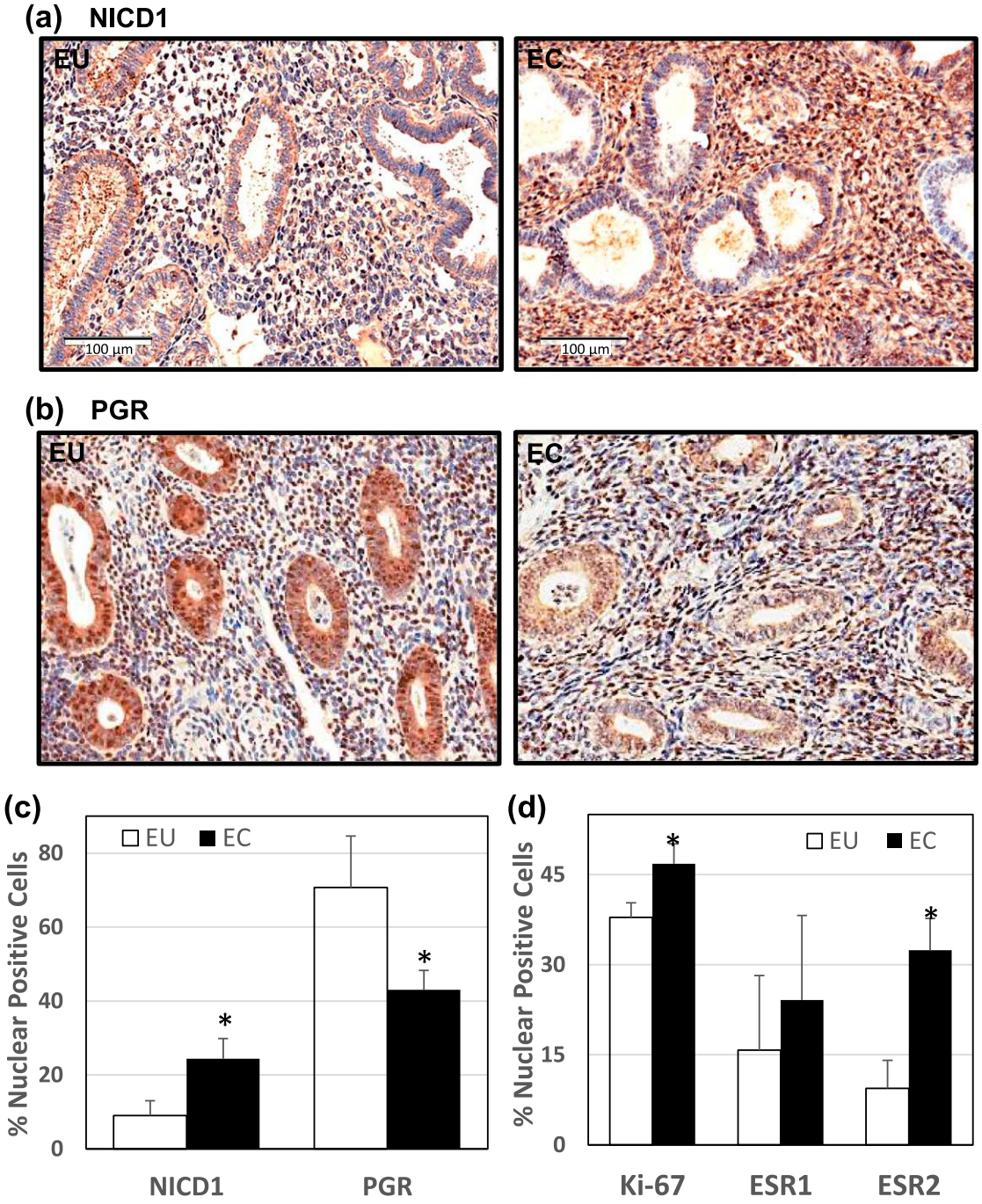

Figure 1. Expression of NICD1 and PGR in EU vs ECs of women with peritoneal endometriosis. Formaldehyde-fixed, paraffin-embedded sections of paired EU and ECs from the same patient (UAMS cohort) were stained with anti-NICD1 or anti-PGR antibodies. Representative images of (a) NICD1 and (b) PGR immunostaining for EU vs ECs are shown. (c) The percentages of nuclear-localized, immunostained stromal cells for NICD1 and PGR were determined by counting the number of immunopositive nuclei over the total number of cells counted per field. Data (mean ± SD) represent analyses of tissue sections from n = 5 patients per group. For each tissue section, three to four random visual fields were counted. (d) Tissue sections were immunostained with anti-Ki67, anti-ESR1, and anti-ESR2 antibodies and analyzed as in c. Data (mean ± SD) are from n = 5 patients per group. For each tissue section, three to four random visual fields were counted. *$P < 0.05$ by Student paired t test between stromal EU and ECs.

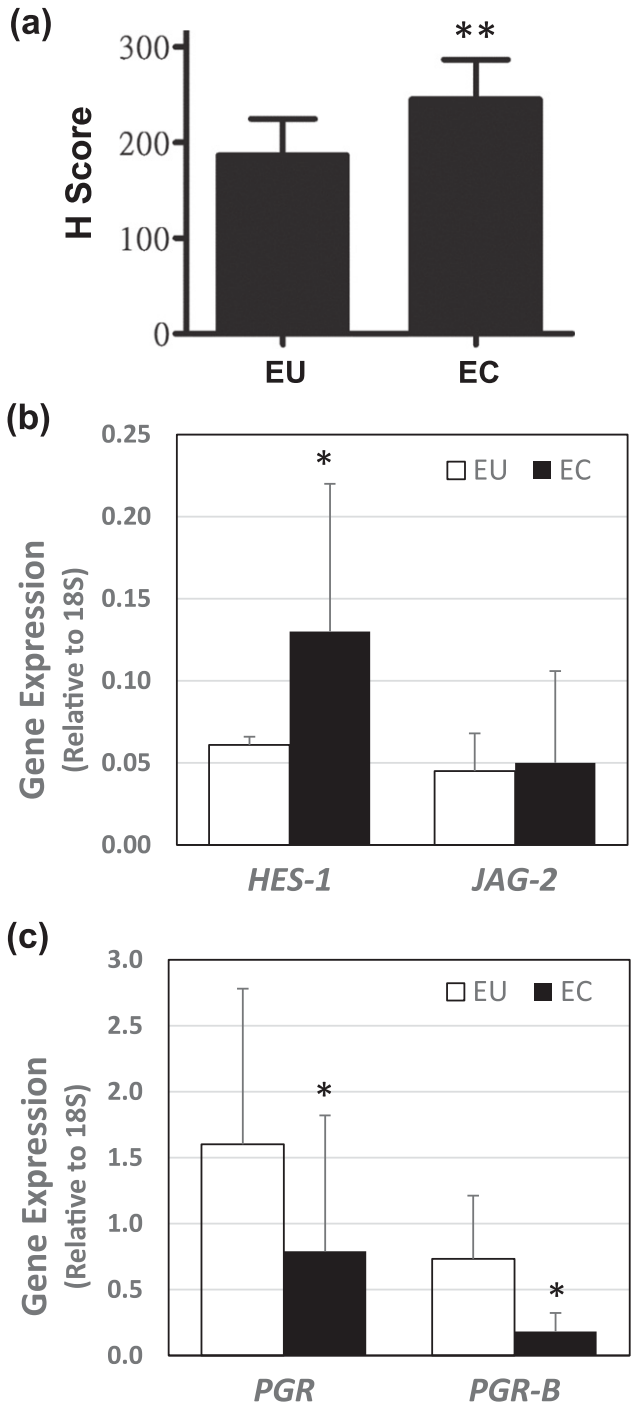

Figure 2. Notch signaling components and PGR expression in EU and ECs. (a) Paraffin-embedded eutopic endometrial tissue (n = 17) and ECs (n = 14) from women with diagnosed endometriosis (Taiwan cohort) were immunostained with anti-NICD1 antibody. Staining scores (0 = none, 1 = weak, 2 = moderate, and 3 = strong) were assigned to immunostained samples. H-scores (mean ± SD) were calculated as described under "Materials and Methods." $^{**}P < 0.0001$ by Student unpaired *t* test between stromal EU and ECs. (b) Stromal cells were isolated from paired EU and ECs of women with endometriosis (n = 6) and subjected to quantitative polymerase chain reaction analyses to quantify Notch signaling components *HES1* and *JAG2* mRNA levels. (c) Stromal cells were isolated from paired EU and ECs of women with endometriosis (n = 6) and subjected to quantitative polymerase chain reaction analyses to quantify total *PGR* and *PGR-B* mRNA levels. For b and c, data (mean ± SD) were normalized to 18S rRNA. $^{*}P < 0.05$ by Student paired *t* test between stromal EU and ECs.

resulted in opposing effects on cell numbers, with DAPT showing a dose-dependent decrease and, conversely, VPA displaying a dose-dependent increase (Fig. 3).

To determine the effects of DAPT and VPA on nuclear NICD1 levels in HESCs and the functional consequences of changes in NICD1 levels, if any, on PGR expression, cells were treated with DAPT (10 μM) or VPA (2 mM) and visualized by immunostaining for NICD1 and PGR in DAPI-stained cells. Representative images of cells treated with DAPT and stained with anti-NICD1 [Fig. 4(a)] and of cells treated with VPA and stained with anti-PGR [Fig. 4(b)] show presence of cells in both cytoplasmic (white arrow) and nuclear (yellow arrow) compartments. The percentages of nuclear-positive cells (*i.e.*, colocalizing with DAPI staining) for NICD1 and for PGR in DAPT-treated [Fig. 4(c)] and VPA-treated [Fig. 4(d)] cells were quantified. DAPT decreased and VPA increased the percentage of cells with nuclear-localized NICD1. Conversely, DAPT increased the percentage of cells with nuclear-localized PGR [Fig. 4(c)]. VPA similarly increased the percentage of cells with PGR localized to the nuclear compartment [Fig. 4(d)]. However, the magnitude of this increase (~40%) was less robust than that attained (~90%) with DAPT treatment [Fig. 4(d) vs Fig. 4(c)].

D. Changes in P Sensitivity of HESCs With Pharmacological Notch Signaling Modification

To evaluate if changes in nuclear PGR levels observed with DAPT or VPA treatments (Fig. 4) alter P sensitivity of endometrial stromal cells, HESCs were exposed to DAPT or VPA (controls are DMSO and PBS, respectively) for 48 hours and then treated with P (1 μM) for another 24 hours. Collected cells were evaluated for cell numbers and for expression of *PGR*, *HES1*, and *DKK1*.

Relative to DMSO-treated cells, cells treated with DAPT or P alone showed comparable reduction in cell numbers [Fig. 5(a)]. Cells cotreated with DAPT and P (DAPT/P) displayed a reduction in cell numbers greater than with either treatment alone [Fig. 5(a)]. In contrast, VPA treatment (2 mM) did not alter cell numbers [Fig. 5(b)] but, when combined with P (VPA/P), increased cell numbers to levels above those obtained with individual and control treatments.

PGR transcript levels were increased by approximately twofold with DAPT or P, relative to DMSO treatment [Fig. 5(c)]. The combination treatments (D/P) increased PGR mRNA levels

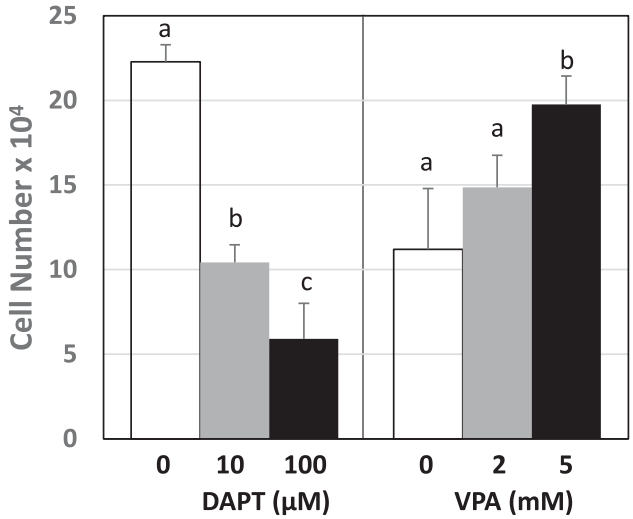

Figure 3. Pharmacological manipulation of Notch-1 signaling altered cell numbers of HESCs. Cells were treated with DAPT (left panel) or VPA (right panel) and respective controls (DMSO for DAPT; PBS for VPA). Cell numbers were determined using a hemocytometer. Data (mean ± SD) are from three independent experiments (n = 4 wells/experiment). Within each treatment group (DAPT or VPA), means with different superscripts differed at $P < 0.05$ as determined by one-way analysis of variance.

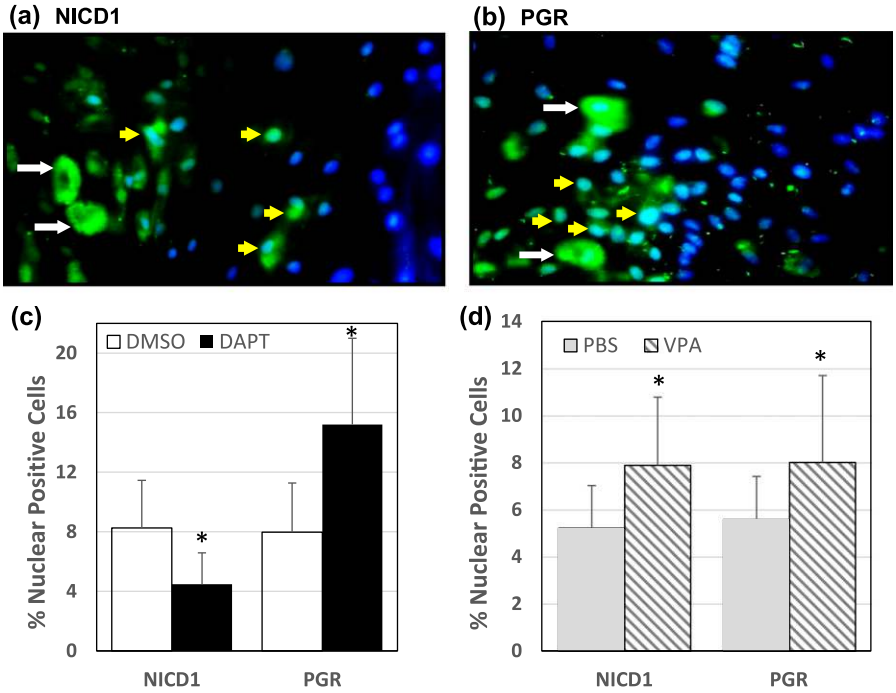

Figure 4. Changes in nuclear-localized NICD1 and PGR in HESCs treated with DAPT or VPA. HESCs were treated with DAPT (10 μM), VPA (2 mM), or corresponding vehicle controls (DMSO for DAPT; PBS for VPA) and immunostained with anti-NICD1 and anti-PGR antibodies followed by incubation with fluorescence-conjugated secondary antibodies. Cells were counterstained with DAPI to identify nuclei. (a) Representative picture of anti-NICD1-immunostained and DAPI-counterstained cells after DAPT treatment. (b) Representative picture of anti-PGR-immunostained and DAPI-counterstained cells after VPA treatment. Nuclei were localized by DAPI (blue staining). For a and b, cytoplasmic-localized (green; white arrow) and nuclear-localized (blue-green; yellow arrow) proteins are shown. Nuclear-localized immunopositive cells for NICD1 and PGR were counted in cells treated with (c) DAPT or (d) VPA (and corresponding controls) and expressed relative to the total number of cells analyzed. Data (mean ± SD) are from two independent experiments, with three individual slides evaluated per treatment group per experiment. $^*P < 0.05$ by Student t test between control and treatment groups for NICD1 and for PGR.

~2.5-fold above those elicited with individual treatments. *HES1* transcript levels were reduced by DAPT and increased by P treatment alone, whereas the combination treatments (D/P) abrogated the individual treatment effects to control levels. *DKK1* transcript levels were increased by P treatment (~3.8-fold) but were unaffected by DAPT alone or when combined with P.

VPA decreased, whereas P tended to increase, *PGR* transcript levels, whereas the cotreatments of VPA and P (V/P) enhanced the reduction of PGR expression to levels below that of control (PBS) or VPA-treated cells [Fig. 5(d)]. As expected, VPA increased *HES1* transcript levels, relative to control cells. P alone increased *HES1* gene expression but did not alter VPA's individual effect in combined treatments. *DKK1* transcript levels were highly induced by P treatment and were reduced by VPA and P (V/P) cotreatment to control levels, although VPA alone showed no effect.

3. Discussion

In a previous study [12], we provided evidence in a mouse model to suggest that Notch-1 signaling activation is associated with progression of endometriosis. In the current study, we extended these findings by our analyses of EU and ECs collected from women with confirmed endometriosis at two different university hospitals. Our results demonstrated induction of

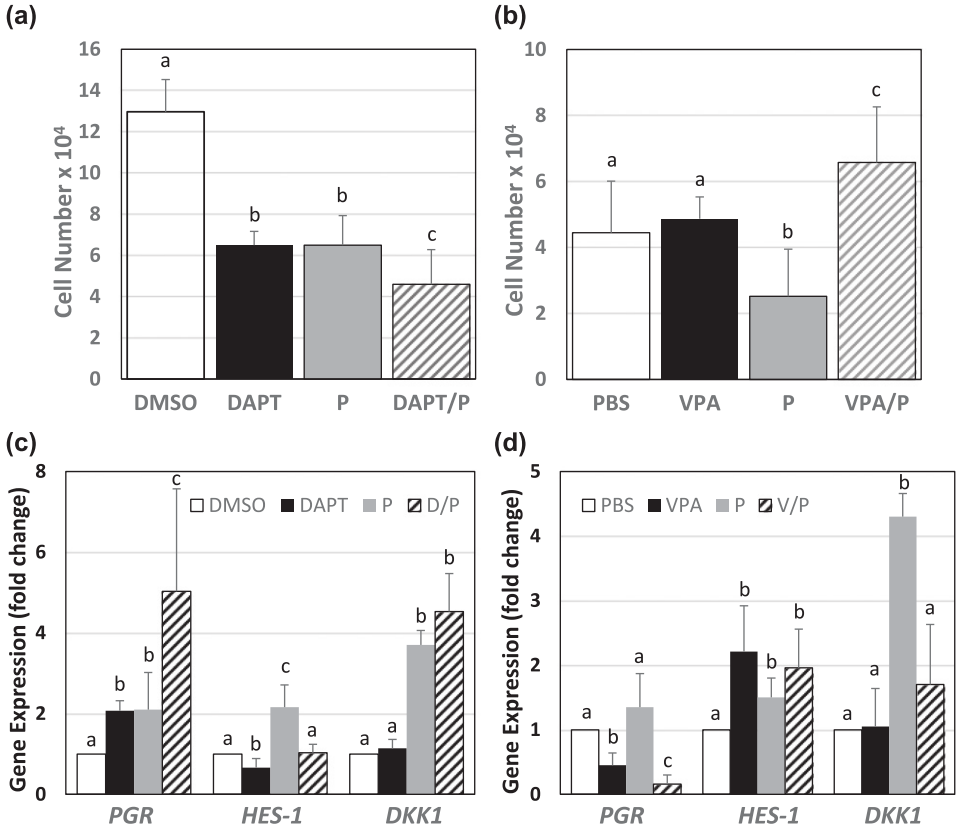

Figure 5. Altered Notch-1 signaling modified responses of HESCs to progestin. HESCs treated with DAPT (10 μM) or VPA (2 mM) in the presence or absence of medroxyprogesterone acetate (P, 1 μM) (DMSO or PBS as control treatments) were evaluated for (a, b) cell numbers and (c, d) gene expression. Data for cell numbers (mean ± SD) are from three independent experiments (n = 4 wells/experiment). Transcript levels of *PGR*, *HES1*, and *DKK1* were evaluated by quantitative polymerase chain reaction; *TATA-Binding Protein* mRNA was used as normalization control. Data (mean ± SD) are expressed as fold change from control cells and were obtained from three independent experiments, with each experiment carried out in duplicate. For a and b, means within each panel showing different superscripts differed at $P < 0.05$ as determined by one-way analysis of variance. For each gene (*PGR*, *HES1*, *DKK1*) in panels c and d, means with different superscripts differed at $P < 0.05$ as determined by one-way analysis of variance.

Notch-1 signaling, assessed by the higher levels of nuclear-localized NICD1 and of *HES1* transcript, in stromal cells of ECs relative to EU. Further, we showed a negative association between Notch-1 activation and PGR expression, coincident with increased proliferation (Ki67) and enhanced *ESR2* expression, in ECs relative to EU. Finally, we confirmed in an endometrial stromal cell line (HESC), by using pharmacological modifiers of Notch-1 signaling, that P sensitivity is optimally sustained with reduction of Notch-1 activation (by DAPT) and, conversely, is robustly attenuated with continued Notch-1 activation (by VPA), due in part to negative regulation by activated Notch of PGR expression. Our results support a role for chronic Notch-1 signaling activation underlying P resistance in women with endometriosis and suggest that the clinical management of the disease may benefit from stringent control of Notch-1 signaling in endometrial stromal cells.

The contribution of Notch signaling to the initiation and progression of endometriosis in women is likely important, although this remains poorly understood. Herein, we report that Notch-1 activation is higher, coincident with decreased PGR/PGR-B expression in ECs vs EU. This inverse association is consistent with a previous report in mice that aberrant activation of uterine Notch-1 signaling caused hypermethylation of the *Pgr* gene, resulting in reduction of uterine PGR expression and infertility [13]. An earlier study from the same group

demonstrated that expression of several Notch signaling components (NOTCH 1/2, NOTCH ligands JAG2 and Delta-like 4) and NICD1 direct target genes *HES5* and *HEY1* were lower in endometria of women with, than without, endometriosis [14]. Although NICD1 levels were not reported, the noted increase in Notch-1 signaling in EU relative to normal endometria does not support the inverse correlation between Notch-1 activation and PGR expression reported in their earlier study [13] and in our present study, and the previously reported decrease in PGR expression in EU relative to endometria of women without endometriosis [10, 11, 34]. Taken together, results suggest that Notch-1 activation provides a regulatory node for P signaling that may differ in normal vs pathological cells.

We found that Notch-1 activation in ECs (relative to EU) was associated with increased ESR2 but not ESR1 levels. ECs are characterized by elevated expression of ESR2 relative to ESR1 [11, 37], and ECs generated in mice from *ESR2*-overexpressing endometrium displayed larger volumes than those from *ESR2*-null endometrium [37]. ESR2 has marked nongenomic actions in ECs, which include induction of inflammation [37], a known trigger of Notch-1 signaling [38, 39]. Inflammation has also been demonstrated to reduce PGR expression and PGR-mediated transcriptional activity [40]. In vascular endothelium, estrogen-mediated regulation of NICD1 levels was abrogated after silencing *ESR2* [41]. Thus, in the context of endometriosis, ESR2's substantial expression and inflammatory effects may tip the balance toward Notch-1 activation and NICD1 downregulation of PGR expression and signaling.

We used pharmacological agents known to interfere with Notch-1 signaling to determine direct effects of Notch-1 activation (VPA) and inhibition (DAPT) *in vitro* on established EC lesion parameters (higher cell numbers, lower PGR expression) by using the HESC. We found that DAPT treatment in a progestogenic environment (*i.e.*, with P treatment) reduced cell numbers and increased PGR expression beyond those obtained with individual P or DAPT exposure and maintained robust PGR transcriptional activity (*e.g.*, *DKK1* transcript levels) in the HESC. Conversely, VPA/P cotreatment increased cell numbers and abrogated PGR-transcriptional activity, coincident with reduction of *PGR* expression. Together, these findings demonstrate that P sensitivity of stromal cells is responsive to alterations in NICD1 levels and suggest that Notch-1 is an upstream regulator of the PGR signaling pathway. Further, because Notch signaling is increased by P in uterine stromal cells [42], in agreement with our findings here that P treatment alone increased *HES1* transcript levels [Fig. 5(c) and 5(d)], presumably by increasing NICD1, results indicate a requirement for the precise maintenance of the PGR/Notch regulatory network, the disruption of which may underlie some, though not all, uterine disorders.

The coincident increase in the percentage of cells with nuclear-localized PGR and with nuclear-localized NICD1 upon VPA treatment [Fig. 4(d)] is unexpected, given that this was accompanied by a reduction in total *PGR* and induction of *HES1* transcript levels [Fig. 5(d)], respectively. We speculate that the above findings may be related to the differential reduction (PGR-B over PGR-A) and/or the preferential nuclear localization (PGR-A over PGR-B) of PGR isoforms subsequent to Notch-1 activation. We used an anti-PGR antibody recognizing both PGR isoforms in these experiments and, hence, are unable to directly address this issue. Nevertheless, we showed herein a disproportionate decrease in *PGR-B* transcript levels relative to total *PGR* transcripts in stroma of EC lesions [Fig. 2(c)]. Moreover, progression to higher endometrial tumor grades was associated with the preferential reduction of focally distributed PGR-B relative to PGR-A [43]. Further studies will address if the latter are similarly observed in the context of endometriosis, using specific anti-PGR-A antibodies. The noted cytoplasmic localization of PGR is not surprising, given its nongenomic actions [44]. Similarly, NICD1 has been localized to membrane, cytoplasmic, and nuclear compartments in many cell types [45], although its detailed compartmental distribution in uterine cells has yet to be described.

Recognized limitations to the current study include the relatively small patient numbers and amounts of tissues, which precluded more extensive analytical analyses, differences in the race/ethnicity of the study population, and variations in the procedures used for tissue identification/collection at the two clinical sites. Despite these, the collective findings

established a consistent inverse association between NICD1 and PGR in ECs. In the UAMS sample cohorts, the higher Ki67 and ESR2 protein levels in ECs relative to EU are consistent with their recognized roles as markers of endometriotic lesions. Moreover, the robust differences in *HES1*, total *PGR*, and *PGR-B* isoform transcript levels found between EU and ECs in the Taiwan cohort suggest that the negative association between NICD1 and PGR may be a feature of more advanced (stage III to IV) endometriosis and, hence, of disease progression.

In conclusion, our findings indicate that aberrant Notch-1 activation may underlie the progestin resistance of ECs of women with endometriosis. Further studies with a larger patient population are indicated to support this functional link.

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