

# Peroxisome proliferator-activated receptor- $\gamma$ coactivator $1\alpha$ -mediated pathway as a possible therapeutic target in endometriosis

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**STUDY QUESTION:** Is a peroxisome proliferator-activated receptor- $\gamma$  coactivator  $1\alpha$  (PGC- $1\alpha$ )-mediated pathway involved in the development of endometriosis?

**SUMMARY ANSWER:** PGC- $1\alpha$  plays critical roles in inflammation and cell proliferation of endometriotic tissues and may be involved in the development of endometriosis.

**WHAT IS KNOWN ALREADY:** Expression levels of PGC- $1\alpha$  are higher in ovarian endometrioma (OE) than normal endometrium (NE). PGC- $1\alpha$  also stimulates aromatase activity and promotes local estrogen biosynthesis in OE.

**STUDY DESIGN, SIZE, DURATION:** This is a case-controlled biological study using endometrial cells and tissues derived from 23 women with, and 10 women without, OE.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Ectopic endometriotic and eutopic endometrial stromal cells (SCs) were isolated and maintained in culture. PGC- $1\alpha$  was either overexpressed in the cells or knocked down using siRNA. The expression of PGC- $1\alpha$  and other factors during endometriosis was examined using real-time PCR and western blotting, cell proliferation was measured using Cell Counting Kit-8 (WST-8) assays and transcriptional activity was assessed using luciferase reporter assays.

**MAIN RESULTS AND THE ROLE OF CHANCE:** PGC- $1\alpha$  overexpression promoted the proliferation of OESCs in a time-dependent manner ( $P < 0.01$  versus control) but not NESCs. PGC- $1\alpha$  stimulated aromatase ( $P < 0.01$  versus control) and interleukin (IL)-6/IL-8 mRNA expression levels ( $P < 0.05$  versus control for each) and led to inhibitor kappa B phosphorylation protein expression and upregulation of the apoptosis inhibitors X-linked inhibitor of apoptosis protein and survivin at mRNA level ( $P < 0.05$  versus control for each). HX531, a selective retinoid-X receptor- $\alpha$  (RXR $\alpha$ ) antagonist, suppressed the PGC- $1\alpha$ -induced cell proliferation ( $P < 0.05$  versus control), aromatase/IL-6/IL-8/survivin mRNA expression ( $P < 0.05$  versus control for each) and transcription reporter activity of PGC- $1\alpha$  in a dose-dependent manner ( $P < 0.01$  versus control). Moreover, HX531 downregulated PGC- $1\alpha$ -induced aromatase-promoter PI.3-II transcripts in OESCs, and PGC- $1\alpha$  knockdown reduced aromatase, IL-6/IL-8 and antiapoptotic factors mRNA expression ( $P < 0.05$  versus control for each). Notably, the Histogram score, which was used for quantifying RXR $\alpha$  status, was markedly higher in OE than in NE tissue ( $P < 0.01$ ).

**LARGE SCALE DATA:** N/A.

**LIMITATIONS, REASONS FOR CAUTION:** Only OE tissues were included in this study, while peritoneal and deep infiltrating endometriotic tissues were not. Therefore, these findings might not be generalized to other types of endometriosis.

**WIDER IMPLICATIONS OF THE FINDINGS:** In OESC, PGC-1 $\alpha$  stimulated cell proliferation and was involved in local estrogen biosynthesis, inflammation and apoptosis, and these effects of PGC-1 $\alpha$  were inhibited by HX531. The suppression of PGC-1 $\alpha$ -induced proliferation by HX531 in OESCs but not NESCs suggests that the PGC-1 $\alpha$ -RXR $\alpha$  axis could play critical roles in promoting endometriosis. This is the first report of a relationship between PGC-1 $\alpha$  and inhibitor of apoptosis proteins in endometriosis. Based on these findings, the PGC-1 $\alpha$ -mediated pathway could represent a potential target in molecular therapy of endometriosis.

**STUDY FUNDING/COMPETING INTEREST(S):** The study is supported in part by Grants-in-Aid for Scientific Research (15 K10681 and 15 K10726) from the Ministry of Education, Culture, Sports, Science, and Technology (Japan). The authors have no conflicts of interest to disclose.

**Key words:** endometriosis / PGC-1 $\alpha$  / estrogen dependence / inflammation / apoptosis

## Introduction

Endometriosis is a common gynecological disease characterized by the presence and growth of endometrium-like tissues at extrauterine sites. Morbidity levels in endometriosis reach ~6–10% in reproductive-age females, and endometriosis causes symptoms such as chronic pelvic pain, dysmenorrhea and infertility, which severely impair patients' health and quality of life (Giudice and Kao, 2004). Although recent studies have investigated the histological origin of endometriosis, the precise etiology of endometriosis remains poorly understood. Currently, hormonal therapy and surgical removal of endometriotic lesions are the established treatments for endometriosis, but hormonal therapy produces adverse side effects, and surgical intervention can be followed by a high recurrence rate and infertility (Vercellini et al., 2008; Seracchioli et al., 2009). Thus, it is crucial to develop endometriosis treatments that are more effective and produce fewer harmful effects than current treatments, and this requires comprehensive elucidation of the molecular pathological properties of endometriotic tissues.

Accumulating evidence indicates that local estrogen production plays key roles in endometriosis pathogenesis (Bulun, 2009). Disease symptoms and endometriotic lesions are frequently relieved after menopause, and thus endometriosis is considered an estrogen-dependent disease. Moreover, the enzyme aromatase, which catalyzes the final step in estrogen biosynthesis, is more highly expressed in endometriotic tissues than in the endometrium (Huhtinen et al., 2012), and estrogen, produced by the abnormally elevated aromatase, promotes the growth of endometriotic tissue (Bulun, 2009; Rizner, 2009; Huhtinen et al., 2012; Colette et al., 2013). Previously, we revealed that peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) coactivator-1 $\alpha$  (PGC-1 $\alpha$ ) contributes to aberrant local estrogen production through aromatase in ovarian endometrioma (OE), but not in endometrium with endometriosis or normal endometrium (NE) without endometriosis (Suganuma et al., 2014). These findings suggest that PGC-1 $\alpha$  represents a promising candidate for novel targeted therapies for endometriosis.

PGC-1 $\alpha$  is a transcription coactivator that interacts with diverse transcription factors involved in various biological responses, including adaptive thermogenesis, mitochondrial biogenesis, oxidative metabolism and steroidogenesis (Liang and Ward, 2006; Cormio et al., 2009; Yazawa et al., 2010). For example, in brown adipose tissue, PGC-1 $\alpha$ , induced by cold exposure, co-activates PPAR $\gamma$  to stimulate adipocyte differentiation (Puigserver et al., 1998); in ovarian granulosa cells, PGC-1 $\alpha$  regulates progesterone production by acting as a coactivator of steroidogenic factor-1 and liver receptor homolog-1 (Yazawa et al., 2010); and in skeletal muscle cells, PGC-1 $\alpha$  affects

glucose intake by downregulating the expression of insulin-sensitive glucose transporter type 4 (Miura et al., 2003). Thus, PGC-1 $\alpha$  is differentially expressed in distinct tissues and functions as a coactivator that interacts with tissue-specific transcription factors. We previously found that PGC-1 $\alpha$  expression was aberrantly elevated in OE and correlated with aromatase tissue localization (Suganuma et al., 2014). However, the functional consequences of PGC-1 $\alpha$  interaction with other transcription factors in endometriosis remain unclear.

Estrogen can potentially act as not only as a proliferative factor but also a proinflammatory and antiapoptotic factor in endometriotic stromal cells (SCs) (Bukulmez et al., 2008; Reis et al., 2013). Interleukin (IL)-6 and IL-8, which are inflammatory cytokines secreted by macrophages and immune cells, are highly expressed in local tissues and the peritoneal fluid of patients with endometriosis (Bergqvist et al., 2001; Harada et al., 2001; Darai et al., 2003), and these cytokines, in turn, promote the proliferation of endometriotic SCs (Iwabe et al., 2000). Moreover, a concomitant overexpression of antiapoptotic factors in SCs is associated with aberrant survival of the cells and thus with their ability to invade the peritoneum and establish an endometriotic implant (Reis et al., 2013), and these effects appear to be exacerbated in endometriosis. Here, we investigated whether PGC-1 $\alpha$  affects endometriotic SC proliferation and examined how PGC-1 $\alpha$  influences estrogen action, inflammation and apoptosis in endometriosis. We propose that PGC-1 $\alpha$  could serve as a molecular target in endometriosis treatments involving the use of compounds that produce transcriptionally inhibitory effects on PGC-1 $\alpha$ .

## Materials and Methods

### Patients and samples

OE specimens were obtained from 23 reproductive-age females (mean age, 36.9 years; range, 24–47 years) who underwent surgery for OE. The endometriosis stages were III ( $n = 7$ ) and IV ( $n = 16$ ) according to the American Society for Reproductive Medicine classification of endometriosis (American Society for Reproductive Medicine, 1997). NE samples were obtained from 10 reproductive-age females (mean age, 35.9 years; range, 24–46 years) who underwent surgery for uterine leiomyoma. None of the patients had received hormone treatment for at least 3 months before surgery. All specimens were at the proliferative phase of the regular menstrual cycle when the surgery was performed. This study was approved by the Institutional Review Board of Kyoto Prefectural University of Medicine (ERB-C-606), and informed consent was obtained from each patient.

**Table 1** List of antibodies used in a study of the PGC-1 $\alpha$ -mediated pathway and endometriosis.

Peptide/Protein target	Manufacturer, catalogue # and batch #	Antigen sequence	Name of the antibody	Species raised, monoclonal or polyclonal	Dilution used	Positive control
Human CD10	Agilent; Clone 56C6; Code M7308	Unknown	Anti-human CD10	Monoclonal mouse	1:80	Human placenta tissue
Human inhibin alpha	Abcam; #ab47720	Unknown	Anti-inhibin alpha antibody (4A2F2)	Monoclonal mouse	1:200	Human ovarian tumor tissue
Human AMH	Proteintech; #14461-I-AP	Human AMH-6 His fusion protein (211–560 amino acid encoded by BC049194)	AMH polyclonal antibody	Polyclonal rabbit	1:25	Mouse ovary tissue
Human I $\kappa$ B	Cell Signaling Technology; #2859	Unknown	Phospho-I $\kappa$ B (Ser32) (14D4)	Monoclonal rabbit	1:1000	HeLa cells treated with TNF- $\alpha$ at 20 ng/ml for 5 min
Human GAPDH	Cell Signaling Technology; #2118	Unknown	GAPDH (14C10)	Monoclonal rabbit	1:1000	-
Human RXR $\alpha$	Proteintech; #21218-I-AP	350 amino acid of human RXRA C-terminal	RXRA antibody	Polyclonal rabbit	1:100	MCF-7 cells
Human RXR $\alpha$ (H10)	Santa Cruz Biotechnology; sc-515929	2–29 amino acid at N-terminal of RXR $\alpha$ human origin	RXR $\alpha$ antibody	Monoclonal mouse	1:50	MCF-7 cells

RXR $\alpha$ , retinoid X receptor alpha; AMH, anti-Müllerian hormone; TNF, tumor necrosis factor.

## Antibodies

All antibodies used in this study are listed in Table 1. The antibody specificity was evaluated by western blotting with the respective positive control. Specificity of the anti-retinoid X receptor (RXR)  $\alpha$  antibody used for immunohistochemistry was also evaluated by comparison of the staining and co-localization with two antibodies raised against different epitopes of the investigated protein (Supplementary Fig. S1).

## Cell isolation and culture

Cells were isolated as described previously (Yamanaka *et al.*, 2012; Suganuma *et al.*, 2014; Takaoka *et al.*, 2018). Briefly, tissues were minced, digested with 2.5% type I collagenase (Sigma-Aldrich, St. Louis, MO, USA) and 15 IU/ml DNase I (Takara Shuzo, Kyoto, Japan) and filtered through a nylon cell strainer. The filtrate was centrifuged with lymphocyte separation solution (Nacalai Tesque, Kyoto, Japan) to remove lymphocytes. Purity (>95%) of all 23 OESCs preparations was confirmed by positive staining for CD10 (Agilent, Santa Clara, CA, USA). Also, the preparation was shown not to be contaminated with granulosa cells and follicular cells by negative staining for inhibin A (Abcam, Cambridge, UK) and anti-Müllerian hormone (Proteintech, Tokyo, Japan; Supplementary Fig. S2). After the cultured primary SCs became sub-confluent, they were collected by treatment with 0.1% trypsin and resuspended in phenol red-free Dulbecco's modified Eagle's medium (DMEM) (Nacalai Tesque) supplemented with 10% dextran-coated, charcoal-treated fetal bovine serum (FBS) and 1% penicillin and 100  $\mu$ g/ml streptomycin; the SCs were then cultured in 6 cm plates for protein extraction, 24-well plates for luciferase assays, 6-well plates for RNA extraction or 96-well plates for WST-8 assays.

## Plasmids and reagents

The plasmid pGL3-ERRE, the pGL3-aromatase-promoter plasmid containing promoters I.3–II (–329/+284; exon I.3 start site designated as +1) and the pSG5-PGC-1 $\alpha$  expression vector were used as described (Suganuma *et al.*, 2014); pSG5-emptyvector and pGL3-basic reporter plasmids were used as controls. HX531, an RXR antagonist, was purchased from Tocris Bioscience (Bristol, UK).

## Transfection

All transfections were performed on sub-confluent cells. Cells were transfected with 0.2  $\mu$ g each of reporter plasmid (pGL3-basic, pGL3-ERRE, pGL3-aromatase-promoter I.3–II) and expression plasmid (pSG5-empty, pSG5-PGC-1 $\alpha$ ) for luciferase assays, 0.1  $\mu$ g of expression plasmid (pSG5-empty, pSG5-PGC-1 $\alpha$ ) for cell proliferation assays, 2.5  $\mu$ g of expression plasmid (pSG5-empty, pSG5-PGC-1 $\alpha$ ) for RNA extraction and 3.75  $\mu$ g of expression plasmid (pSG5-empty, pSG5-PGC-1 $\alpha$ ) for western blotting analysis. Lipofectamine LTX (Life Technologies, Carlsbad, CA, USA) was used for transfection as per the manufacturer's protocol. Cells were cultured in phenol red-free DMEM supplemented with 10% dextran-coated, charcoal-treated FBS and then used for analyses after 48–72 h incubation.

## Luciferase assay

Luciferase assays were performed using the ONE-Glo<sup>TM</sup> EX Luciferase Assay System and a GloMax 20/20 Luminometer (Promega, Madison, WI, USA) as per the manufacturer's protocol.

## Exon I-specific RT-PCR

Aromatase exon I-specific RT-PCR analysis was performed as described (Kinoshita and Chen, 2003). Briefly, RT-PCR was performed using each forward primer of aromatase exons I.3, PII and II with a reverse primer of exon II to amplify exons I.3, PII and II. GAPDH primers were used as an internal control. Primer sequences are listed in [Supplementary Table S1](#). RT-PCR was conducted using Premix Taq (Takara Shuzo) and a GeneAmp PCR 9700 (Applied Biosystems, Foster City, CA, USA) and the following thermal cycling conditions: stage 1, 10 s at 98°C; stage 2, 35 cycles of 10 s of melting at 98°C, followed by DNA synthesis for 30 s at 54°C and 60 s at 72°C. PCR products were electrophoresed on 1.5% agarose/Tris-borate-EDTA gels, and the integrated band intensities in ethidium bromide-stained gels were analyzed using a ChemiDoc XRS+ system (Bio-Rad, Hercules, CA, USA).

## Cell proliferation and cytotoxicity assays

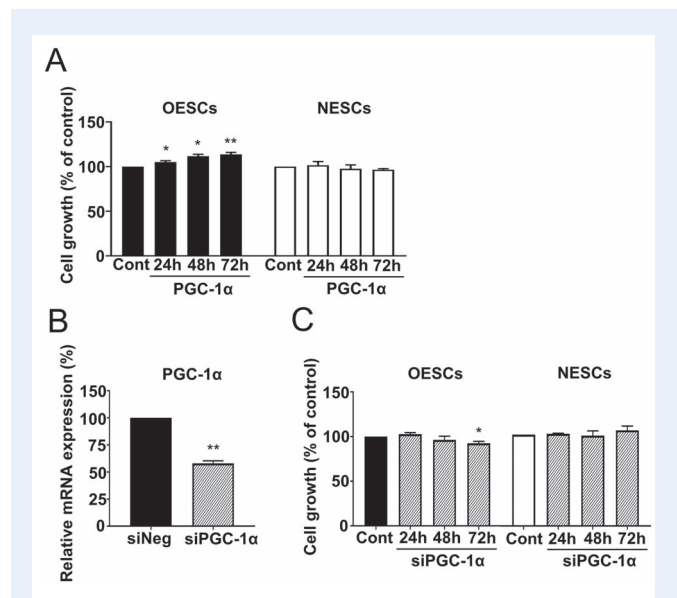
OESCs and NESCs were cultured and maintained in 96-well plates for 24 h and then transfected with pSG5-empty/PGC-1 $\alpha$ . After HX531 treatment for 24–72 h, viable cells were quantified using a modified WST-8 assay (Nacalai Tesque); 10  $\mu$ L of the WST-8 reagent (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulphophenyl)-2H-tetrazolium, monosodium salt) was added to each well and the plates were incubated for 2 h at 37°C, after which 450/655 nm absorbance was measured using a microplate reader. Compound cytotoxicity was examined using the CytoTox-Fluor cytotoxicity assay (Promega). Fluorescence was measured at 485Ex/520Em by using a SpectraMax M2e instrument (Molecular Devices, San Jose, CA, USA) after 24–72 h treatment with/without HX531.

## mRNA extraction, cDNA preparation and real-time PCR analysis of human tissue

Total RNA was extracted from excised OE and NE tissues by using a RNeasy Mini Kit (QIAGEN, Hilden, Germany) as per manufacturer instructions, and RNA concentrations were determined by measuring the ultraviolet (260/280 nm) absorption with a Nano Drop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Next, 1  $\mu$ g of each RNA sample was used to prepare cDNA by using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) and a GeneAmp PCR 9700, and the cDNA was used for real-time PCR and exon I-specific RT-PCR. Real-time PCR was performed using a Step One Real-Time PCR system (Applied Biosystems) and this protocol: stage 1, 60 s at 95°C; stage 2, 40 cycles of 3 s of melting at 95°C, followed by DNA synthesis for 30 s at 60°C. Quantitative real-time PCR was conducted using a Power SYBR Green kit. The PCR mixture (20  $\mu$ L) contained 1  $\mu$ L of cDNA samples prepared from tissues and cells and 0.3  $\mu$ M of each primer for the target genes. The sequences of the primers used are listed in [Supplementary Table S1](#). Threshold cycle (Ct) values were calculated using the  $\Delta\Delta$ Ct method (Yamanaka et al., 2012).

## Western blotting

Cells in primary culture were washed twice in PBS and lysed in radioimmunoprecipitation assay buffer (Nacalai Tesque). Cell lysates (20  $\mu$ g) were heated in sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 25% glycerol, 10% 2-mercaptoethanol,



**Figure 1** PGC-1 $\alpha$  effect on human OESC and NESC proliferation analyzed using WST-8 assays. **(A)** Effect of PGC-1 $\alpha$  overexpression on OESC ( $n = 6$ )/NESC ( $n = 5$ ) proliferation. **(B)** Real-time PCR analysis of PGC-1 $\alpha$  knockdown and **(C)** the effect of the knockdown on OESC ( $n = 5$ )/NESC ( $n = 4$ ) proliferation; cells were transfected with control or PGC-1 $\alpha$  siRNA (siPGC-1 $\alpha$ ). Results are expressed as percentages of control values (Cont) and presented as means  $\pm$  SEMs. Statistical analysis was performed using Student's *t*-test and repeated measures ANOVA, followed by multiple comparisons using Dunnett's procedure. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

0.05 mM phenylmethanesulfonyl fluoride and 0.004% bromophenol blue), separated on 10% e-PAGE gels as per the manufacturer's protocol (Atto Corp, Tokyo, Japan) and transferred onto Immobilon-Blot<sup>®</sup> polyvinylidene difluoride (PVDF) membranes (Bio-Rad). The membranes were blocked in PVDF Blocking Reagent (Toyobo) for 1 h and then incubated with antibodies (rabbit anti-Phospho-I $\kappa$ B $\alpha$  (#2859) and rabbit anti-GAPDH (#2118), Cell Signaling, Danvers, MA, USA) at 4°C overnight. After washing, the membranes were incubated with the secondary antibody (anti-rabbit IgG, HRP-linked antibody (#7074), Cell Signaling) for 1 h at room temperature. The staining signal was developed using Chemi-Lumi One Super (Nacalai Tesque) and analyzed using a ChemiDoc XRS+ system with Image Lab software (Bio-Rad).

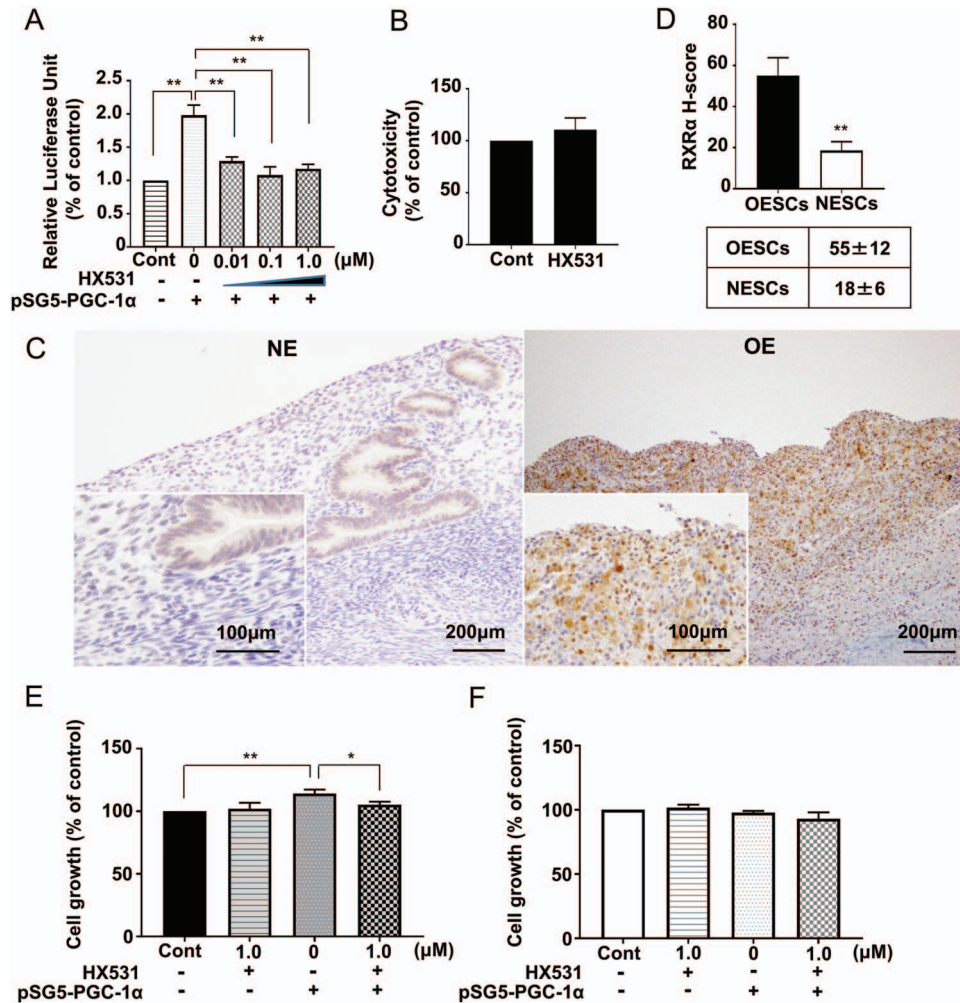
## RNA interference

PGC-1 $\alpha$ -specific siRNA (s21395) and negative control siRNA (siNeg) (control #1) targeting known genes were purchased from Thermo Fisher Scientific (Silencer Select siRNAs<sup>®</sup>). Cells were transfected with siRNAs by using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) as per the manufacturer's protocol and used for experiments after 72 h. After incubation, the cells were processed for each analysis.

## Immunohistochemistry

OE and NE specimens were immunohistochemically stained as described (Mori et al., 2011). OE tissue was pathologically





**Figure 2** PGC-1 $\alpha$  and retinoid-X receptor- $\alpha$  (RXR $\alpha$ ) signaling in human OE. (A) Luciferase activity of ERRE promoter in PGC-1 $\alpha$ -overexpressing OESCs ( $n = 3$ ); the assay was performed in triplicate. (B) Cytotoxicity of HX531 (1.0  $\mu$ M) in OESCs. (C) RXR $\alpha$  immunohistochemical staining in NE/OE specimens. (D) H-scores of RXR $\alpha$  immunoreactivity in NE ( $n = 8$ )/OE ( $n = 9$ ) specimens. HX531 effect on the proliferation of (E) OESCs ( $n = 5$ ) and (F) NESCs ( $n = 5$ ) analyzed using WST-8 assays. Results are expressed as percentages of control values (Cont) and presented as means  $\pm$  SEMs. Statistical analysis was performed using Student's  $t$ -test and repeated measures ANOVA, followed by multiple comparisons using Dunnett's procedure. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

diagnosed with the presence of endometriotic SC by CD10 staining and the hemosiderin deposit by haematoxylin-eosin staining (Supplementary Fig. S3). Anti-RXR $\alpha$  antibody was used at 200  $\mu$ g/ml. The immunostaining intensities of OE and NE were evaluated using the Histogram score (H-score), a semiquantitative index calculated as described previously (Yamanaka *et al.*, 2012). Briefly, two independent observers (H.K. and O.T.) evaluated approximately 500 cells/slide and scored them as follows: 3  $\times$  percentage of strongly staining cells, + 2  $\times$  percentage of moderately staining cells and + percentage of weakly staining cells. The H-score was calculated as the mean of the two scores.

### Statistical analysis

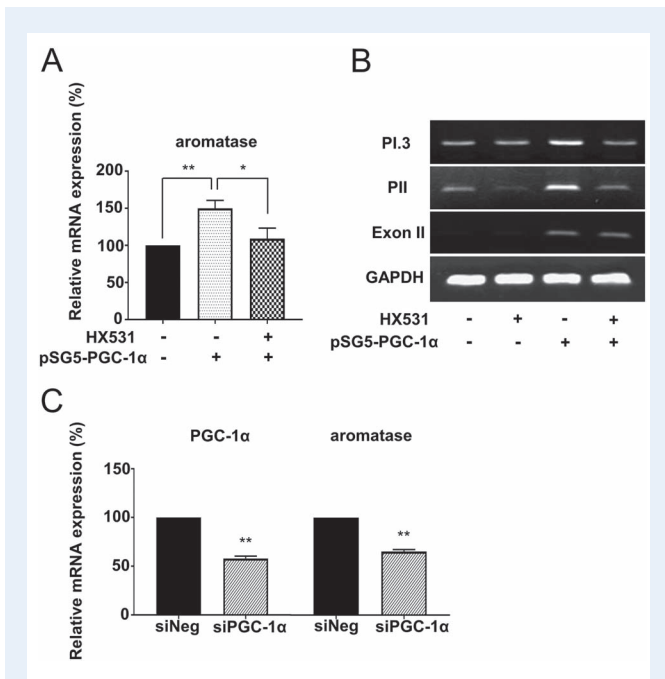
Real-time PCR results and immunohistochemical H-scores were compared between two groups using Student's  $t$ -test. Cell proliferation

assay results, PGC-1 $\alpha$  overexpression induced mRNA expression and HX531 effect were analyzed using repeated measures ANOVA, followed by multiple comparisons using Dunnett's procedure. All analyses were performed using Prism software v7 (Graph Pad Software Inc., San Diego, CA, USA). Data are presented as mean  $\pm$  standard error of the mean (SEM). All assays for each experiment were performed in triplicate.  $P < 0.05$  was considered statistically significant.

## Results

### PGC-1 $\alpha$ promoted proliferation of OESCs but not NESCs

To examine whether PGC-1 $\alpha$  affects the proliferation of endometriotic SCs, we performed WST-8 cell proliferation assays. PGC-1 $\alpha$

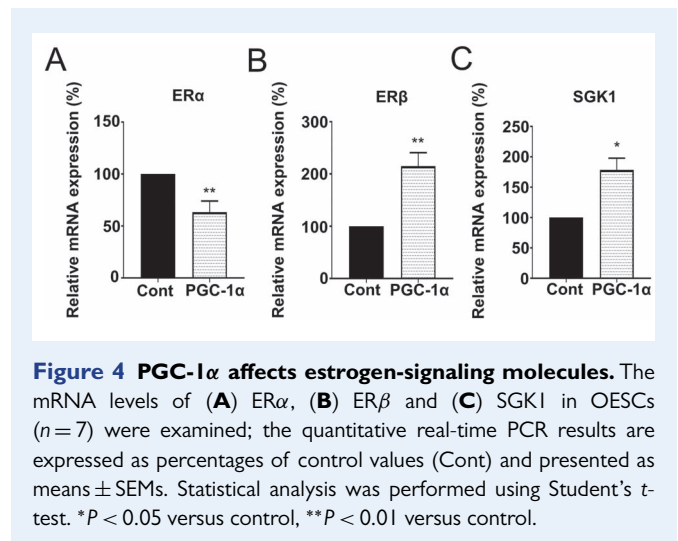


**Figure 3 PGC-1 $\alpha$  regulates aromatase expression in human SCs.** (A) Quantitative real-time PCR analysis of aromatase mRNA levels in OESCs ( $n=6$ ) treated with HX531 (1.0  $\mu$ M). (B) Aromatase exon I-specific RT-PCR. PCR products detected for exons PI.3, P.II, II and GAPDH were 333, 234, 169 and 224 bp in size, respectively. (C) Real-time PCR analysis of PGC-1 $\alpha$  knockdown and the effect of the knockdown on aromatase mRNA expression in OESCs ( $n=4$ ); cells were transfected with siNeg or siPGC-1 $\alpha$  for 72 h. Results are expressed as percentages of control values and presented as means  $\pm$  SEMs. Statistical analysis was performed using Student's  $t$ -test and repeated measures ANOVA, followed by multiple comparisons using Dunnett's procedure. \* $P < 0.05$ , \*\* $P < 0.01$  versus control.

overexpression caused a time-dependent and statistically significant increase in proliferation in OESCs but not NESCs (Fig. 1A). In addition, all individual experiments confirmed promotion of significant cell proliferation by PGC-1 $\alpha$  overexpression (Supplementary Fig. S4). We also conducted loss-of-function experiments; real-time PCR analysis confirmed siRNA-mediated PGC-1 $\alpha$  knockdown (Fig. 1B), and WST-8 assay results showed that the knockdown inhibited the proliferation of OESCs but not NESCs (Fig. 1C).

### HX531, an RXR $\alpha$ antagonist, inhibited PGC-1 $\alpha$ -induced transcriptional activity in OESCs

Next, to identify compounds that might exert transcriptionally inhibitory effects on PGC-1 $\alpha$ , we performed luciferase reporter assays. Previously, our chromatin immunoprecipitation results showed that PGC-1 $\alpha$  was recruited to a region containing the nuclear receptor half-site 5'-AGGTCA-3' (ERRE) (Suganuma et al., 2014). Here, we confirmed that PGC-1 $\alpha$  induced the luciferase transcriptional activity of ERRE in OESCs and then screened various compounds; we found that 72 h treatment with the RXR $\alpha$  antagonist HX531 inhibited PGC-1 $\alpha$ -induced ERRE activity (Fig. 2A). PPAR $\gamma$  agonist and antagonist did not show an inhibitory effect on PGC-1 $\alpha$ -induced ERRE activity



**Figure 4 PGC-1 $\alpha$  affects estrogen-signaling molecules.** The mRNA levels of (A) ER $\alpha$ , (B) ER $\beta$  and (C) SGK1 in OESCs ( $n=7$ ) were examined; the quantitative real-time PCR results are expressed as percentages of control values (Cont) and presented as means  $\pm$  SEMs. Statistical analysis was performed using Student's  $t$ -test. \* $P < 0.05$  versus control, \*\* $P < 0.01$  versus control.

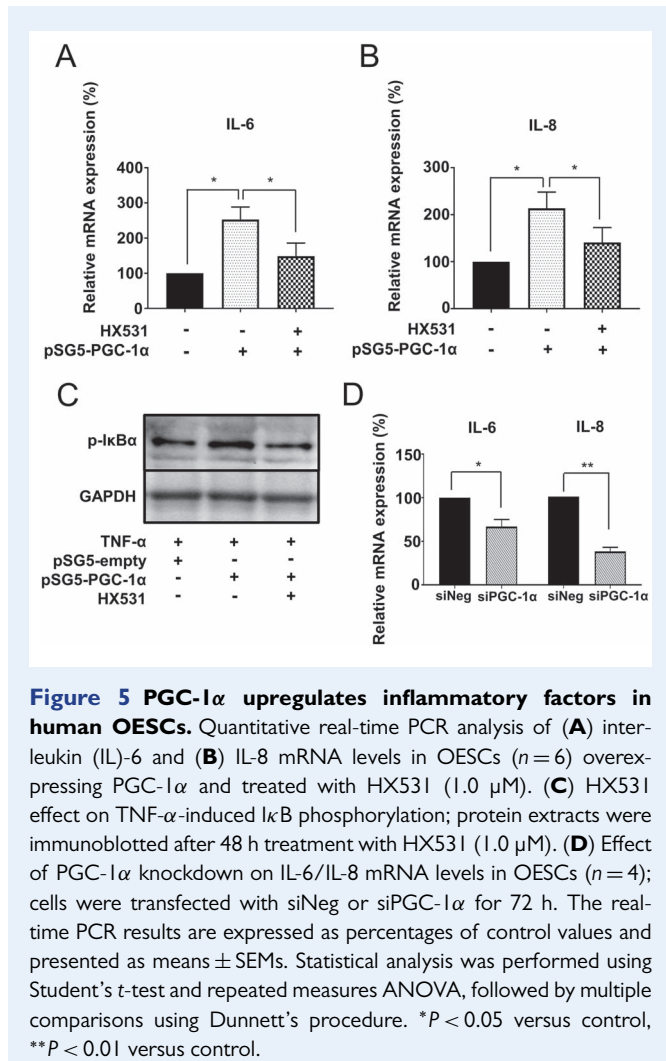
(Supplementary Fig. S5). CytoTox-Fluor assay results showed that HX531 was not toxic to OESCs (Fig. 2B). Immunohistochemical analysis of OE and NE specimens further revealed high RXR $\alpha$  expression in both the nucleus and the cytoplasm in OE specimens (Fig. 2C), and RXR $\alpha$  immunostaining intensity was markedly higher in OESCs than NESCs (Fig. 2D). Notably, HX531 suppressed PGC-1 $\alpha$ -induced OESC proliferation but not NESC proliferation. (Fig. 2E and F). Immunohistochemistry of peritoneal endometriotic explants and deep infiltrating endometriosis showed positive immunoreaction of SCs to PGC-1 $\alpha$  (Supplementary Fig. S6).

### PGC-1 $\alpha$ enhanced aromatase expression and promoter activity in OESCs

We previously demonstrated that PGC-1 $\alpha$  enhanced aromatase expression and promoter activity in OESCs (Suganuma et al., 2014). Accordingly, PGC-1 $\alpha$  increased aromatase mRNA levels (Fig. 3A), and this induced expression was markedly suppressed by HX531 (Fig. 3A). Next, we performed aromatase exon I-specific RT-PCR. The aromatase gene contains nine translated exons (II–X) and multiple untranslated exon I $s$ . In endometriotic tissues, PI.3 and P.II are mainly used to regulate aromatase mRNA expression (Zeitoun et al., 1999). In accord with our report (Suganuma et al., 2014), we confirmed that the transcripts of the aromatase promoters PI.3, P.II and exon II were induced by PGC-1 $\alpha$ , and HX531 downregulated PGC-1 $\alpha$ -stimulated PI.3–II aromatase promoter transcripts in OESCs (Fig. 3B). Moreover, as reported previously, we did not detect mRNA expression of aromatase exon I $s$  or II in EESCs and NESCs, regardless of PGC-1 $\alpha$  expression (Suganuma et al., 2014). Furthermore, the results of loss-of-function experiments showed that siRNA-mediated PGC-1 $\alpha$  knockdown substantially reduced aromatase-coding mRNA levels in cells (Fig. 3C).

### PGC-1 $\alpha$ enhanced expression of estrogen receptor $\beta$ and SGK1 in OESCs

To understand the regulation of PGC-1 $\alpha$ -induced estrogen receptor (ER) signaling, we examined the expression of ER $\alpha$  and ER $\beta$  and of serum and glucocorticoid-regulated kinase (SGK1), a potent downstream transcriptional regulator of ER $\beta$  that helps exacerbate



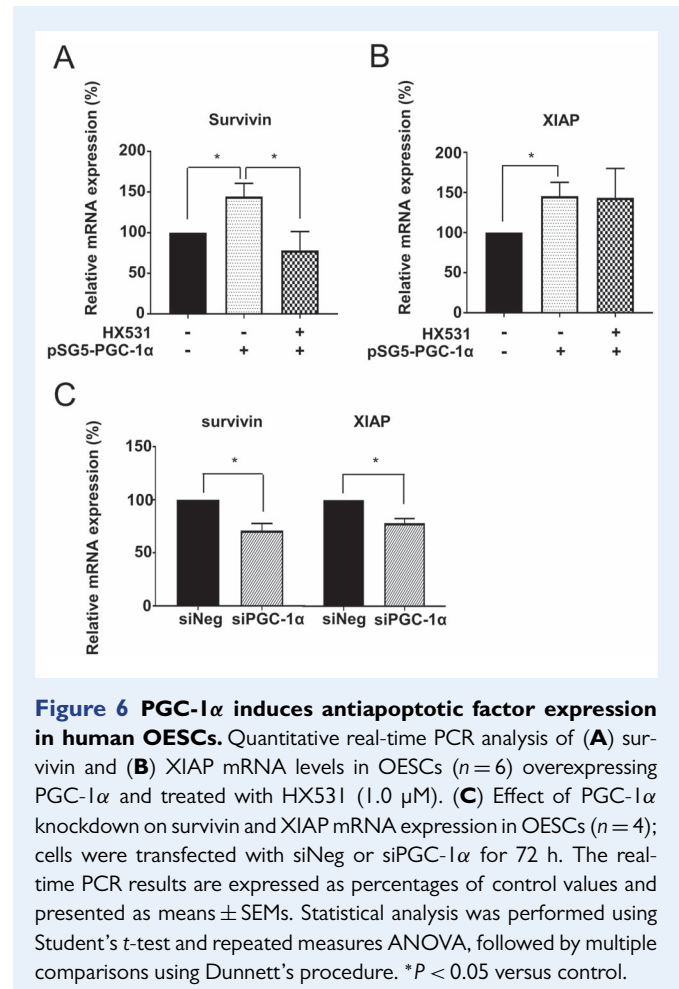
endometriosis. Real-time PCR results showed that PGC-1 $\alpha$  increased ER $\beta$  and SGK1 expression in OESCs (Fig. 4B and C) but reduced ER $\alpha$  expression (Fig. 4A).

### PGC-1 $\alpha$ elevated inflammatory cytokine expression in OESCs

We performed real-time PCR analysis to assess how PGC-1 $\alpha$  affects inflammatory cytokines in endometriosis. PGC-1 $\alpha$  elevated IL-6 and IL-8 mRNA levels in OESCs, and HX531 attenuated these responses (Fig. 5A and B). The nuclear factor-kappa B (NF- $\kappa$ B) and inhibitor kappa B (I $\kappa$ B) complex could help elevate IL-6/IL-8 levels in endometriosis (Kim *et al.*, 2012); I $\kappa$ B phosphorylation results in NF- $\kappa$ B activation and this would lead to IL-6/IL-8 elevation (Gonzalez-Ramos *et al.*, 2010). Here, western blotting revealed that in OESCs, PGC-1 $\alpha$  induced I $\kappa$ B phosphorylation under tumor necrosis factor (TNF)- $\alpha$  stimulation, and HX531 inhibited this induced phosphorylation (Fig. 5C). Moreover, siRNA-mediated PGC-1 $\alpha$  knockdown decreased IL-6/IL-8 mRNA levels in OESCs (Fig. 5D).

### PGC-1 $\alpha$ upregulated IAPs in OESCs

Lastly, examination of PGC-1 $\alpha$  effects on inhibitor of apoptosis proteins (IAPs; antiapoptotic factors) in OESCs revealed that PGC-1 $\alpha$



overexpression increased survivin and X-linked inhibitor of apoptosis protein (XIAP) mRNA levels (Fig. 6A and B). Intriguingly, HX531 markedly attenuated PGC-1 $\alpha$ -induced expression of survivin but not XIAP (Fig. 6A and B). However, siRNA-mediated PGC-1 $\alpha$  knockdown reduced the expression of both antiapoptotic factors in OESCs (Fig. 6C).

## Discussion

We demonstrated here for the first time that PGC-1 $\alpha$  stimulated the proliferation of OESCs but not NESCOs. We also showed that PGC-1 $\alpha$  induced ER $\beta$  and SGK1 expression in OESCs as well as aromatase, as we previously reported (Suganuma *et al.*, 2014). Our results further indicated that PGC-1 $\alpha$  upregulated the inflammatory cytokines IL-6 and IL-8 and promoted apoptosis resistance in OESCs. Moreover, we identified a compound that produced transcriptionally inhibitory effects on PGC-1 $\alpha$  in OESCs; HX531, an RXR antagonist, inhibited PGC-1 $\alpha$ -induced cell proliferation and PGC-1 $\alpha$ -elevated expression of aromatase, IL-6/IL-8 and survivin in OESCs. Thus, we propose that PGC-1 $\alpha$  could play critical roles in endometriosis development and might represent a novel molecular target for endometriosis treatment.

PGC-1 $\alpha$ , a transcriptional coactivator that interacts with diverse transcription factors, can potentially function in numerous biological responses, including adaptive thermogenesis, mitochondrial biogenesis and glucose and fatty acid metabolism. PGC-1 $\alpha$  is highly expressed



in tissues that are enriched in mitochondria and where oxidative metabolism is active, such as brown adipose tissue, heart and skeletal muscle (Puigserver et al., 1998). PGC-1 $\alpha$  is expressed at lower levels in breast and colon cancer tissues than in normal tissues (Jiang et al., 2003; Feilchenfeldt et al., 2004; Watkins et al., 2004), but is highly expressed in uterine endometrial cancer tissues (Ren et al., 2015). Previously, we showed that PGC-1 $\alpha$  expression was higher in OE than NE by using specimens from patients with or without endometriosis, and the expression was significantly correlated with aromatase expression in OE (Suganuma et al., 2014). Here, cell proliferation assays revealed that PGC-1 $\alpha$  promoted the proliferation of OESCs but not NESCs, whereas PGC-1 $\alpha$  knockdown reduced the proliferation. All individual experiments confirmed promotion of significant cell proliferation by PGC-1 $\alpha$  overexpression (Supplementary Fig. S4). These findings indicate that PGC-1 $\alpha$  potentially represents a molecular target for endometriosis therapy.

We next sought to identify compounds that could control PGC-1 $\alpha$  function in endometriosis. To our knowledge, no ligands for PGC-1 $\alpha$  are currently available. PGC-1 $\alpha$  interacts with PPAR $\gamma$  in a ligand-independent manner (Puigserver et al., 1998). PPAR $\gamma$  binds to peroxisome proliferator response elements (PPREs), which are located in promoters and contain two copies of the core motif 5'-AGGTCA-3'. Interestingly, our previous chromatin immunoprecipitation analysis revealed PGC-1 $\alpha$  recruitment to the nuclear half-site ERRE, 5'-AGGTCA-3', in OESCs. Thus, we used the ERRE site with a luciferase reporter system to identify compounds that exhibit agonistic/antagonistic activities toward PGC-1 $\alpha$  in OESCs. We initially examined a PPAR $\gamma$  agonist and antagonist, pioglitazone and GW9662; the use of these compounds is widely accepted because several lines of evidence have indicated their clinical or preclinical efficacy in diabetes treatment and reduction of macrovascular events in diabetes patients (Dormandy et al., 2005). However, pioglitazone and GW9662 showed no effects on transcription (Supplementary Fig. S5). We next focused on compounds that affect RXR $\alpha$ , because PPARs form heterodimers with RXR $\alpha$ , which leads to binding of the PPRE site and transcriptional coactivation by PGC-1 $\alpha$  (Mangelsdorf et al., 1995; Giguere, 1999; Delerive et al., 2002; Gronemeyer et al., 2004). Luciferase reporter assay results showed that among the compounds tested here, HX531 produced transcriptionally inhibitory effects on PGC-1 $\alpha$  in OESCs. Moreover, immunohistochemical analysis revealed that RXR $\alpha$  expression was higher in OE than NE, and, intriguingly, the subcellular localization of RXR $\alpha$  in OE specimens was mostly consistent with that of PGC-1 $\alpha$ . Previous evidence has also indicated overlapping tissue distribution of PGC-1 $\alpha$  and RXR $\alpha$  (Chambon, 1996; Puigserver et al., 1998). However, further investigation is required to elucidate PGC-1 $\alpha$  interaction with RXR $\alpha$  in endometriosis. Here, HX531 suppressed PGC-1 $\alpha$ -induced proliferation of OESCs, but not NESCs, which suggests that the PGC-1 $\alpha$ -RXR $\alpha$  axis could play critical roles in promoting endometriosis.

Estrogen production plays a key role in endometriosis (Kitawaki et al., 1997; Bulun et al., 2005; Huhtinen et al., 2012). Previously, we reported that aromatase is more highly expressed in endometriotic tissue than in NE and PGC-1 $\alpha$  stimulated aromatase activity (Kitawaki et al., 1997; Suganuma et al., 2014). In contrast, some studies reported that aromatase was not expressed in endometriotic tissues (Colette et al., 2009). This discrepancy in aromatase expression between these

studies might be due to differences in specificity of antibody or the biopsy specimens investigated. The aromatase gene contains nine translated exons (II–X) and multiple copies of untranslated exon I. Each exon I is regulated by its own promoter located immediately upstream of exon I, and each promoter is regulated through distinct mechanisms, which leads to tissue-specific aromatase expression. Our exon I-specific RT-PCR confirmed previous findings indicating that PI.3–II of the aromatase gene represents the main promoter in OESCs (Zeitoun et al., 1999). HX531 attenuated PGC-1 $\alpha$ -induced elevation of expression and PI.3–II transcripts of aromatase in OESCs, and the levels of aromatase-coding mRNA were also markedly decreased in PGC-1 $\alpha$ -knockdown cells. These results suggest that PGC-1 $\alpha$  represents a key therapeutic target for controlling estrogen production by regulating aromatase in endometriosis.

Locally upregulated estrogen binds to ERs in endometriosis and thus stimulates estrogen-dependent growth (Bulun, 2009). ER $\beta$  expression was shown to be higher in OE than NE, and signaling through ER $\beta$  was found to play critical roles in endometriosis establishment and maintenance (Xue et al., 2007; Juhasz-Boss et al., 2011; Han et al., 2012). Here, PGC-1 $\alpha$  altered expression patterns to cause higher expression of ER $\beta$  than ER $\alpha$  in OESCs. ER $\beta$  was previously shown to directly bind to ER $\alpha$  promoter and repress ER $\alpha$  expression, leading to a state of progesterone resistance in endometriosis (Bulun et al., 2012). However, it remains unknown whether PGC-1 $\alpha$  directly interacts with ER isoforms and affects progesterone resistance. Bulun et al. (2012) recently found that SGK1 is a transcriptional target in ER $\beta$ -mediated regulation and this helps promote endometriotic SC survival. Interestingly, our study showed that PGC-1 $\alpha$  also increased SGK1 expression in OE. Collectively, these findings suggest that PGC-1 $\alpha$  accelerates ER $\beta$ -mediated signaling in endometriosis.

IL-6 and IL-8 are representative inflammatory cytokines that are highly elevated in the peritoneal fluid of patients with endometriosis and in OESCs (Harada et al., 1997). Harada et al. have also demonstrated that TNF- $\alpha$  upregulates IL-6 and IL-8 production through NF- $\kappa$ B activation in OESCs (Sakamoto et al., 2003; Yamauchi et al., 2004; Takai et al., 2013). Recently, activation of the NF- $\kappa$ B and I $\kappa$ B complex was shown to potentially help elevate IL-6/IL-8 levels in endometriosis (Kim et al., 2012). Thus, previous evidence indicates that NF- $\kappa$ B activity in endometriotic tissues stimulates inflammation and cell proliferation (Kim et al., 2011; Zhang et al., 2011). Here, we found that PGC-1 $\alpha$  upregulated IL-6/IL-8 expression in OESCs, and HX531 attenuated this elevated expression, and further that PGC-1 $\alpha$  activated I $\kappa$ B phosphorylation and HX531 suppressed this response in OESCs. These results suggest that PGC-1 $\alpha$  could exert inflammatory effects by regulating the NF- $\kappa$ B and I $\kappa$ B complex in endometriosis, which disagrees with the only previous related report showing that in skeletal muscle cells PGC-1 $\alpha$  inhibited IL-6 expression in the presence of TNF- $\alpha$ . Further investigation is necessary to clarify the underlying mechanism because no study to date has shown PGC-1 $\alpha$  association with the NF- $\kappa$ B and I $\kappa$ B complex in endometriosis.

Apoptosis—programmed cell death—plays a critical role in maintaining tissue homeostasis by eliminating excess cells. Endometriotic cells show diminished spontaneous apoptosis and resistance to apoptotic stimuli such as cytokines (Dmowski et al., 2001; Nishida et al., 2005), and impaired apoptotic mechanisms contribute to the abnormal survival of these cells in ectopic sites. The inability of endometriotic cells to undergo normal apoptosis is associated with



their increased expression of antiapoptotic factors. Previous studies have demonstrated that IAP expression is higher in OESCs than NESCs, and that survivin, an IAP-family representative, plays a critical role in the apoptosis susceptibility of endometrial SCs (Taniguchi *et al.*, 2009; Uegaki *et al.*, 2015). We found that PGC-1 $\alpha$  enhanced survivin and XIAP mRNA levels in OESCs, and further that HX531 attenuated PGC-1 $\alpha$ -induced expression of survivin but not XIAP in OESCs. To our knowledge, this is the first report of a relationship between PGC-1 $\alpha$  and IAPs in endometriosis. Previously, in skeletal muscle cells, PGC-1 $\alpha$  was shown to enhance hypoxia-inducing factor (HIF)-1 $\alpha$ -dependent gene expression by increasing mitochondrial oxygen consumption (O'Hagan *et al.*, 2009). Interestingly, the HIF-binding site (hypoxia-responsive element) is located in the survivin promoter, and hypoxia has recently been shown to play critical roles in endometriosis development and progression (Lee and Tsai, 2017). Thus, the crosstalk between PGC-1 $\alpha$  and apoptotic factors under hypoxia warrants investigation.

As mentioned above, PGC-1 $\alpha$  plays an important role in various biological responses. PGC-1 $\alpha$  is differentially expressed in distinct tissues and functions as a coactivator interacting with tissue-specific factors. Because RXR $\alpha$  interacts with other nuclear receptors, inhibiting RXR $\alpha$  functions for treatment of endometriosis might inhibit other unexpected functions. Therefore, targeting PGC-1 $\alpha$  directly is more likely to be beneficial for treatment of endometriosis. However, it is also important to inhibit PGC-1 $\alpha$  functions locally and not systemically in order to avoid major side effects. In this study, we used NESCs as control samples from women with uterine leiomyoma. Ideally, the control group would comprise age-matched fertile women with no uterine pathology. To minimize the effect of the leiomyoma on adjacent endometrial SC, patients with subserosal uterine leiomyoma were chosen because subserosal leiomyoma induced less inflammatory effects on endometrium than submucosal and intramural leiomyoma (Miura *et al.*, 2006). We also used OESC to investigate the function of PGC-1 $\alpha$  and not peritoneal endometriotic explants, which are more common than OE. We also confirmed that PGC-1 $\alpha$  was highly expressed in peritoneal and deep infiltrating endometriotic specimens by immunostaining (Supplementary Fig. S6). We believe that these results could be applicable to peritoneal and deep infiltrating endometriosis. Further investigation is needed to confirm our findings.

In conclusion, we demonstrated that PGC-1 $\alpha$  could play critical roles in cell proliferation, estrogen signaling, inflammation and antiapoptosis in endometriosis. Interestingly, HX531, a recognized RXR $\alpha$  antagonist, was identified as a compound that exerts transcriptionally inhibitory effects on PGC-1 $\alpha$ ; HX531 suppressed PGC-1 $\alpha$ -induced cell proliferation and expression of aromatase, IL-6 and IL-8. Moreover, survivin, a key molecule in apoptosis resistance in endometriosis, might be regulated by PGC-1 $\alpha$ . Thus, the PGC-1 $\alpha$ -mediated pathway represents a potential target in molecular therapy for endometriosis.

## Supplementary data

Supplementary data are available at *Human Reproduction* online.

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## Authors' roles

H.K., T.M. and J.K. designed the study. H.K., H.O., H.M., F.I., A.K., Y.T., K.A., E.M., T.S. and Y.T. carried out the experiments. H.K., T.M., H.O. and Y.T. contributed to the interpretation of the results. H.K. and T.M. wrote article. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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## Conflict of interest

The authors have nothing to declare.

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