Hypoxia induces expression of COX-2 through the homeodomain transcription factor CDX1 and orphan nuclear receptor SHP in human endometrial cells

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ABSTRACT: Endometriosis, the presence of ectopic endometrial tissue outside the uterine cavity, is a common disease affecting women during their reproductive years. The aim of this study was to identify the molecular mechanism of transcriptional regulation of inflammatory cyclooxygenase-2 (COX-2) gene during endometriosis by hypoxia. Hypoxia induced COX-2 expression in endometrial cells together with the induction of the orphan nuclear receptor SHP and intestinal-specific transcription factor Caudal-related transcription factor 1 (CDX1). Hypoxia-inducible factor (HIF)-1α was responsible for SHP induction mediated by a hypoxia. In addition, we observed that ectopic expression of CDX1 enhanced COX-2 gene expression in hypoxia-dependent fashion. Additionally, we evaluated that induction of CDX1 by hypoxia was mediated by SHP. Expression of COX-2, CDX1, SHP and HIF-1α mRNA in hypoxia-treated human endometrial cells were significantly higher than normal control cells. These results suggest that the SHP and CDX1 expression increased by hypoxia play an active role in inducing inflammatory COX-2 expression in the pathogenesis of endometriosis.

Key words: endometriosis / ovarian cancer / hypoxia / CDX1 / COX-2 / SHP

Introduction

Hypoxia is considered a physiological stress response that leads to a stimulation of angiogenesis in endometrial tissues during the premenstrual period (Donnez et al., 1998). In the late secretory phase and menstruation, high expression of pro-inflammatory cytokines and the induction of hypoxia originating from vasoconstriction of the spiral arteries have been noted (Gazvani and Templeton, 2002; Wu et al., 2007). The expression of hypoxia-inducible factor (HIF)-1α is also detected during these phases in normal endometrium. The intensity of HIF-1α protein expression increases over the course of the secretory phase and is maximal during menstrual phase in glandular and stromal cells in the functional layer of endometrium (Critchley et al., 2006; Goteri et al., 2010). Based on these clinical observations and the pathophysiological characteristics shown, we postulate that expression of HIF-1α in endometriotic tissues may be up-regulated and thereby plays a role in the development of endometriosis. There is general agreement that endometriosis is a chronic pelvic inflammatory process, characterized by enhanced numbers of activated peritoneal immune cells and pro-inflammatory factors (Lebovic et al., 2001; Gazvani and Templeton, 2002; Lousse et al., 2008). In particular, increased concentrations of prostaglandins (PGs) (De Leon et al., 1986; Wu et al., 2002) and leukotrienes (Yamaguchi and Mori, 1990) have been found in the peritoneal fluid of endometriosis patients. These are the major constituents of a group of biologically active oxygenated fatty acids known as eicosanoids and have been implicated in various inflammatory diseases (Peters-Golden and Henderson, 2007; Smyth et al., 2009). In endometriosis, they appear to play an important role in disease-associated pain (Koike et al., 1992; Wu et al., 2007), essentially treated with non-steroidal anti-inflammatory drugs (Vercellini et al., 2008). These inflammatory mediators, particularly PGs, may also be directly involved in the pathogenesis of endometriosis (Wu et al., 2007), as recent in vitro studies have demonstrated that improved synthesis is involved in enhancing
proliferation, while inhibiting apoptosis, increasing both angiogenesis and immunosuppression (Sales and Jabbour, 2003). Unlike many other biologically active molecules, the final products of eicosanoids are not stored preformed, but are synthesized de novo through a cascade of enzymes that are mainly regulated by transcriptional control. The cyclooxygenase (COX) pathway leads to the formation of PGs and involves COX and PG synthase activities. Each of these reactions can be rate-limiting and catalyzed by several enzymatic isoforms (Murakami and Kudo, 2004). In contrast to constitutive isoforms, inducible isoforms are typically undetectable under normal physiological conditions, but may be expressed at high levels following stimulation by pro-inflammatory cytokines (Lindstrom and Bennett, 2004). This has been reported particularly for type II secretory phospholipase A2, cyclooxygenase-2 (COX-2) and microsomal PGE synthase-1 (Lindstrom and Bennett, 2004).

Concentrations of PG estradiol (PGE2) in peritoneal fluid are higher in women suffering from endometriosis than in disease-free women (Karck et al., 1996). Much of the endometriotic pain is thought to be due to high levels of PGE2 (Allen et al., 2005). COX-2 is more abundantly expressed in ectopic endometria than in the eutopic endometria during similar phases of the menstrual cycle in women (Ota et al., 2001; Chishima et al., 2002). COX-2 is expressed in both glandular epithelium and stroma of endometriotic tissues in women (Ota et al., 2001; Chishima et al., 2002). Cytokines increase COX-2 expression in endometriotic stromal cells (Wu et al., 2007). PGE2 modulates the expression of steroidogenic acute regulatory protein and aromatase in endometriotic stromal cells and thereby regulates estrogen metabolism (Ebert et al., 2005), which is primarily mediated through the PGE2 receptor (Zeitoun and Bulun, 1999). Moreover, inhibition of COX-2 prevents establishment of endometriosis (Matsuzaki et al., 2004; Ozawa et al., 2006), decreases size and number of endometriotic tissues (Efstathiou et al., 2005; Laschke et al., 2007) and prevents neoangiogenesis in endometriotic implants (Laschke et al., 2007) in different animal models for study of endometriosis.

Considering that Caudal-related transcription factor 1 (CDX1) can promote inflammation and be regulated by hypoxia, we hypothesized that low cellular oxygen tension in endometriotic stromal and epithelial cells might up-regulate CDX1 expression to increase the inflammation of endometrial cells. Herein, we provide compelling evidence to demonstrate that hypoxia induces the expression of small heterodimer partner (SHP) and CDX1 in endometrial cells, which normally does not express SHP and CDX1. Hypoxia-induced SHP gene expression is mediated via HIF-1α binding to the functional hypoxia-responsive element (HRE) in the SHP promoter. Our findings provide a new etiological insight into the development of endometriosis as well as a molecular framework for designing new therapeutic strategies for this disease.

Materials and Methods

Subjects

Ten women (ages, 26–39 year; mean age, 31.4 ± 3.7) with endometriosis (I/II) were enrolled in this study. These women treated for infertility and/or pelvic pain, were found to have endometriosis during laparoscopy, and had not received any anti-inflammatory, or any other hormonal treatment during a period of at least 3 months before surgery. All patients enrolled in this study were in proliferative phases of the menstrual cycle. Specimens were obtained from peritoneum or ovarian surface. Control tissues were obtained from 10 women. The collection of uterine endometrium was carried out by endometrial sampling procedure using sterile, disposable endometrial suction curettes (Z-sampler, Zinnanti, Chatsworth, CA, USA). Endometriotic biopsies were immediately placed at 4°C in sterile Hank’s balanced salt solution containing 100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin. All cases were diagnosed and staged as endometriosis according to the revised classification of The American Society of Reproductive Medicine and confirmed by histopathological characterizations. Written informed consent was obtained from all of the patients and the Institutional Review Board at Pusan National University Hospital approved this protocol.

Endometrial cell culture

The tissues were treated with 0.25% collagenase type I in Dulbecco’s modified Eagle’s medium (DMEM) at 37°C for 30 min in a shaking water bath and subsequently the cells were passed through 100- and 70-μm sieve (BD Falcon, Bedford, MA). The cells retained on the sieve were collected separately and used for the experiments as epithelial cells: normal endometrial epithelial (EE) cell, epithelial cell of ovarian red lesion (ORE) and epithelial cell of ovarian black lesion (OBE). After sedimentation, the supernatant was transferred to a new tube and cells were collected by centrifugation. The cells were maintained and proliferated in DMEM supplementing with 10% fetal calf serum (FCS), in a humidified atmosphere of 5% CO2 at 37°C. The stromal cell-rich supernatant was placed in a culture flask, and cells were allowed to adhere for 20 min and then washed with the medium. Adherent stromal cells were cultured as monolayers in flasks with DMEM/F-12 (1:1) (Sigma) containing antibiotics/antimycotics, 5 μg/ml insulin (Sigma) and 10% FCS: normal endometrial stromal (ES) cell, stromal cell of ovarian red lesion (ORS) and stromal cell of ovarian black lesion (OBS). Purity of cultures was determined morphologically after hematoxylin and eosin (H&E) staining. Stromal cells were fusiform, but more rounded than fibroblasts. Cells were also evaluated immunohistochemically using monoclonal antibodies to human cytokeratin, vimentin, von Willebrand factor and CD45.

Construction of reporter plasmids and expression vectors

pGL2/COX-2 promoter (−327 to +59) construct was generously provided by Dr Min DS (Pusan National University, Korea). Two nested S′-end deletion constructs of the COX-2 promoter were generated by PCR using the plasmid pGL2/COX-2 (−327 to +59) as the template. Two nested S′-end deletion constructs were generated using the restriction enzymes Mull and BglII site was added to the 5′-end of COX-2-antisense. Two constructs generated by PCR were amplified with the same 3′-oligonucleotide (COX-2 antisense) and the corresponding specific 5′-oligonucleotide: COX-2 -80 (5′-AGC CGT AGG TTC TCT CGG TTA-3′); COX-2 -20 (5′-CGC AGG CTTGG AAA GAA ACA-3′); COX-2-antisense (5′-GGG AGG ATC TCG TGT CCG AGG AGT TC-3′). The PCR product was double-digested with Mull and BglII cloned into pGL2 luciferase reporter (Promega).

pcDNA3/CDX1 and pGL3B/hCDX1 promoter (−1630 to +12) construct was a kind gift from Yoo MA (Pusan National University, Korea). Two nested 5′-end deletion constructs were generated using the restriction enzymes MulI. A BglII site was added to the 5′-end of COX-2-antisense. Two constructs generated by PCR were amplified with the same 3′-oligonucleotide (COX-2 antisense) and the corresponding specific 5′-oligonucleotide: COX-2 -80 (5′-CGC AGT AGG TTC TCT CGG TTA-3′); COX-2 -20 (5′-CGC AGG CTTGG AAA GAA ACA-3′); COX-2-antisense (5′-GGG AGG ATC TCG TGT CCG AGG AGT TC-3′). The PCR product was double-digested with Mull and BglII cloned into pGL2 luciferase reporter (Promega).

pcDNA3/HA/CDX1 was constructed by inserting PCR fragments of open-reading frame (ORF) into EcoRI and Xhol digested pcDNA3/HA expression vector. All plasmids were confirmed by automatic sequencing analysis.
Cell line culture and hypoxia treatment

Human ovarian cell lines SKOV3 (a gift from Kim KH, Pusan National University, Korea) and A2780 (a gift from Choi YH, Dongeui University, Korea) were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY, USA) and 1% (v/v) penicillin-streptomycin (Gibco-BRL) at 37°C in a humidified atmosphere containing 5% CO₂ and fed every 2 days. To achieve a hypoxic atmosphere, cells were placed on hypoxia chamber supplying 2% O₂ and 5% CO₂ at 37°C for indicated times.

RNA preparation and RT–PCR

Total RNA from SKOV3 and A2780 cells were prepared using Trizol reagent (Invitrogen) according to the manufacturer’s recommendation. The cDNA was synthesized from 2 μg of total RNA with AMV Reverse Transcriptase (Promega) using a random hexamer (Bioneer, Korea) at 42°C for 1 h. The PCR primer for CDX1 gene amplification were: 5′-AGGACAAGT ACCGCCGTGCTCTA-3′ (sense), 5′-CTATGGGAGA AACCTCCTTTC C-3′ (antisense); for SHP gene amplification: 5′-AGCCTGTGCACTCT ATCGCACTGC-3′ (sense), 5′-CAAGCGAGCTGTGCAATGG ACTTGT-3′ (antisense); for COX-2 gene amplification: 5′-CC TTCCCTGCTGTGCTATT G-3′ (sense), 5′-CTGCGGCTCCTGTATGA TCT-3′ (antisense); and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene amplification: 5′-GTTGCTCTTCGCATTCAC-3′ (sense), 5′-TCTCTTCTCCTGTGCTTTG-3′ (antisense). The cDNAs were amplified by PCR under the following conditions: 32 cycles of denaturation at 95°C for 40 s, annealing at 57°C for 40 s and extension at 72°C for 1 min each: a low-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1), a high-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl) and a LiCl immune complex washing buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Precipitates were washed two times with Tris–EDTA buffer and then used in PCR assays to determine the binding sequences. For PCR assays, precipitates were extracted three times with 1% SDS, 0.1 M NaHCO₃. Elutions were pooled and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified; 1 μl from 30 μl of DNA extract was reserved for 32 cycles of PCR amplification.

Transient transfection assay and luciferase assay

A2780 and SKOV3 cells were seeded in a 24-well culture plate and transfected with reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using JetpeI (polyplus-transfection). The pcDNA3.1/HisC plasmid DNA was added to achieve the same total amount of plasmid DNA per transfection. After 48 h of transfection, the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and lysed with lysis cell culture lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity, and all assays were performed at least in triplicate.

The construction of small interference RNA (siRNA) targeted to CDX1 and HIF-1α was carried out according to the instruction manual provided by the manufacturer (Bioneer, Korea). For the construction of CDX1 siRNA (the sense; 5′-ACAAGAAGUGCCCGAGUA-3′; the antisense; 5′-UCAACGAGGCGAUAUCUCUG-3′) and HIF-1α siRNA (sense: 5′-GGAAGAAGCUAAUCCAC-3′, antisense: 5′-UUUGGAUUAGUUCUCUCU C-3′) oligonucleotides were synthesized.

Chromatin immunoprecipitation assay

A2780 cells were collected and cross-linked with 1% formaldehyde at 37°C for 10 min and then rinsed with ice-cold PBS twice and centrifuged for 5 min at 2000 g. Cells were then resuspended in 0.2 ml lysis buffer [1% sodium dodeyl sulfate (SDS), 10 mM EDTA, 50 mM Tris–Cl, pH 8.1, 1 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)] and sonicated nine times for 10 s each, followed by centrifugation for 10 min. Supernatants were collected and diluted in buffer (1.1% Triton X-100, 0.01% SDS, 1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl, pH 8.1) followed by immunoprecipitating with 60 μl protein G-agarose and 2 μg sheared salmon sperm DNA for 30 min at 4°C. Immunoprecipitation was performed overnight at 4°C with the anti-HA antibody (Roche). After immunoprecipitation, 60 μl protein G-agarose with 2 μg sheared salmon sperm DNA was added, and the incubation was continued for another hour. Precipitates were washed sequentially in the following three washing buffers for 5 min each: a low-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), a high-salt immune complex washing buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl) and a LiCl immune complex washing buffer (250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl, pH 8.1). Precipitates were washed two times with Tris–EDTA buffer and then used in PCR assays to determine the binding sequences. For PCR assays, precipitates were extracted three times with 1% SDS, 0.1 M NaHCO₃. Elutions were pooled and heated at 65°C for at least 6 h to reverse the formaldehyde cross-linking. DNA fragments were purified; 1 μl from 30 μl of DNA extract was reserved for 32 cycles of PCR amplification.

SDS–polyacrylamide gel electrophoresis and western blots

Cells were suspended in a lysis buffer (150 mM NaCl, 1% NP-40, 1 mM EDTA, 50 mM Tris and pH 7.5) containing protease inhibitors, and quickly sonicated on ice. Protein concentrations were measured using a commercially Protein Assay (Bio-Rad). Whole cell lysates were subjected to SDS–polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore) by semidry electroblotting. The membranes were then incubated for 2 h at room temperature with anti-CDX1 (AB4122, Chemicon), anti-SHP (sc-150, Santa Cruz), anti-COX-2 (sc-1745, Santa Cruz), anti-HA (Roche) and anti-β-actin (A 2066, Sigma) in tris-buffered saline tween-20 (TBST) supplemented with 1% nonfat dry milk. After washing three times with cold TBST, the blotted membranes were incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature. After washing three times with cold TBST, the bands were detected using an enhanced chemiluminescence system (Amersham).

PGE₂ enzymeimmunoassay immunoassay

PGE₂ level in cell supernatants was measured using commercially available kits (900-001, ENZO) according to the manufacturer’s protocol. The limit of PGE₂ assay was 39–2500 pg/ml.

Statistical analysis

Statistical analyses were carried out by unpaired or paired t-test as appropriate. All data are reported as means ± SD. P-value of <0.05 was considered significant.

Results

Hypoxia increases mRNA expression of COX-2 and SHP in human primary ES and EE cells

Hypoxia exerts different biological effects on cell growth, cell transformation and gene expression. In particular, hypoxia performs a function in tumor promotion, and higher levels of hypoxia have been reported in patients suffering from endometriosis. Although hypoxia is the principal etiologic agent associated with endometriosis and alterations in COX-2 expression are observed during endometriosis,
the molecular mechanisms underlying the hypoxia-mediated overexpression of COX-2 remain to be determined.

In an effort to assess the effects of hypoxia on the modulation of COX-2 expression, we examined human primary endometrial cells from 10 patients, which are normal ES cell, ORS, stromal cell of ovarian black lesion (OBS), normal EE cell, ORE and OBE. Endometriosis was initially diagnosed by H&E staining based on the identification of endometrial glands and stroma. Immunohistochemical staining for CD10 was performed on endometriotic lesions for further histologic confirmation (Supplementary data, Fig. S1).

COX-2 expression in OBS cells was higher than ES and ORS. As predicted, nuclear factor-kappa B (NF-κB) p65 mRNA expression was consistently increased with COX-2 expression (Fig. 1A). In previous results, the orphan nuclear receptor SHP induced COX-2 expression and also acted as a coactivator for NF-κB. Based on these, we examined whether SHP was increased together with COX-2 and NF-κB induction. As shown in Fig. 1A and B, SHP expression in ORE and OBE cells in addition to ORS and OBS cells were higher than ES and EE cell, respectively. Hypoxia treatment significantly increased the gene expression of COX-2 and SHP in every tested primary cell compared with untreated cases (Fig. 1A and B). Because ectopic endometriotic lesions showed a significant increase of toll-like receptor (TLR)3 and TLR4 mRNA expression compared with corresponding eutopic tissues, we also analyzed the TLR expression as a marker protein of endometriosis.

**Hypoxia increases COX-2 expression at the transcriptional level**

To confirm the hypoxia-mediated COX-2 induction in endometrial cells, we examined the mRNA and protein expression of COX-2 by hypoxia treatment in cultured ovarian cell lines. When compared with the untreated cells, hypoxia increased the levels of steady-state COX-2 mRNA and protein within 1 and 3 h, respectively, in A2780 and SKOV3 cells (Fig. 2A). These results demonstrate that COX-2 expression is stimulated by hypoxia treatment in a time-dependent manner in ovarian cells. A2780 and SKOV3 cells were transiently transfected with human COX-2 promoter (−327 to +59) construct for examining transcriptional activation by hypoxia. As shown in Fig. 2B, hypoxia treatment increased the activity of the COX-2 promoter in a time-dependent manner in the A2780 and SKOV3 cells. Luciferase activity of statistical analysis and mRNA levels, using GAPDH mRNA on the same blot for normalization, revealed an apparent good correlation. These indicate that hypoxia increases COX-2 expression at the transcriptional level dependent on the COX-2 promoter activation. In addition, COX-2-derived PGE2 biogenesis increased by hypoxia in A2780 cells, but it was abolished by treatment with celecoxib, a highly selective COX2 inhibitor (Fig. 2C). Since PGE2 is an important inflammation modulator in various tissues including endometriosis, the result indicates the biological role of COX-2 under hypoxia in endometrial cells.

**Hypoxia increases COX-2 expression via the induction of SHP**

Next, in order to determine whether hypoxia increases COX-2 expression via the transcriptional regulation of SHP, A2780 and SKOV3 cells were transfected with the human SHP promoter reporter and then maintained in the presence or absence of hypoxia treatment. SHP promoter activity increased in a time-dependent manner in two ovarian cells by hypoxia (Fig. 3A). We confirmed that hypoxia induced an increase in the level of SHP mRNA in both cells when compared with the untreated cells (Fig. 3A, right panel).

Furthermore, in order to investigate the involvement of HIF-1α in hypoxia-mediated SHP induction, we used the transactivation assay of SHP gene promoter. In Fig. 3B, A2780 and SKOV3 cells were transfected with the indicated amounts of the expression vectors encoding for HIF-1α [hemagglutinin (HA) tagged] along with the human SHP promoter in the presence or absence of hypoxia treatment. The results demonstrated that HIF-1α increased SHP promoter activity in a dose-dependent fashion. In order to further confirm the induction of the SHP gene by HIF-1α directly, A2780 cells were transiently transfected with HIF-1α expression vector or with empty vector as a control, then subjected to western blot assay. As shown in Fig. 3B,

![Figure 1](https://academic.oup.com/molehr/article-abstract/17/11/710/1028073)
The ectopic HIF-1α expression significantly stimulated SHP protein levels. Western blotting of anti-HA showed HA-HIF1α protein expression. These results show that HIF-1α directly increases the hypoxia-mediated SHP induction in cells.

To examine whether the transactivation of the SHP promoter by HIF-1α expression results from direct DNA binding activity of HIF-1α, we applied the chromatin immunoprecipitation (ChIP) assay. Isolated DNA was subjected to PCR both before (marked ‘Input’ in Fig. 3C) and after ChIP using primer sets designed to amplify the region of the SHP promoter harboring the HIF-1α binding site. ChIP assay demonstrates that HIF-1α directly binds to SHP promoter (−1332 to −1328) (Fig. 3C). However, there is no amplified PCR product at the lane of pcDNA/HA transfected control group. The result suggests that HIF-1α increases the SHP expression at the transcriptional level through direct DNA binding.

Next, in order to obtain additional confirmation on HIF-1α function in the hypoxia-mediated SHP expression, loss of HIF-1α expression studies were conducted using an siRNA approach. The knock-down of HIF-1α attenuated the transactivation activity of the SHP promoter in the presence of hypoxia (Fig. 3D). In order to verify the level of SHP expression along with siRNA directed against HIF-1α, RT–PCR and western blot assays were conducted. The knock-down of HIF-1α resulted in a reduction in the levels of SHP mRNA and protein expression (data not shown). These results show that HIF-1α performs a critical function in hypoxia-induced SHP expression.

**CDX1 is associated with hypoxia-induced COX-2 expression through SHP transactivation**

In previous report, the SHP-mediated COX-2 induction resulted from CDX1 transactivation. Therefore, to investigate the role of SHP on CDX1 expression in ovarian cells by hypoxia, promoter assay, RT–PCR and western blot were performed. For elucidating the effect of SHP on the regulation of human CDX1 promoter activity, A2780 and SKOV3 cells were transiently transfected with an expression vector encoding SHP expression plasmid and CDX1 promoter-reporter. The results shown in Fig. 4A illustrated that SHP increased the luciferase activity derived from the CDX1 promoter in a dose-dependent manner of SHP transfection. Additionally, to determine whether hypoxia-induced COX-2 expression can be
mediated by CDX1, A2780 and SKOV3 cells were transfected with the indicated amounts of the expression vectors encoding CDX1 and maintained in the presence or absence of hypoxia treatment. As shown in Fig. 4B, ectopic CDX1 expression increased the hypoxia-induced COX-2 promoter transactivation.

Based on the results presented in Fig. 4A and B, we confirmed mRNA and protein induction of CDX1 and COX-2 by SHP expression and hypoxia. A2780 and SKOV3 cells were transfected with SHP expression vector or with empty vector as control in the presence or absence of hypoxia treatment and detected the mRNA and protein levels of CDX1 and COX-2 by RT–PCR and western blotting, respectively. As shown in Fig. 4C, ectopic expression of SHP significantly increased mRNA and protein production of CDX1 and COX-2. In addition, hypoxia further enhanced the SHP-induced increase of CDX1 and COX-2. These results indicate that CDX1 induction regulated by SHP was associated with an increase of COX-2 expression in a cascade reaction.

**COX-2 induction by hypoxia requires CDX1-mediated transactivation**

To gain additional confirmation of CDX1 activation in COX-2 expression, loss-of-function studies were performed using a siRNA approach. Knock-down of CDX1 gave rise to reduce the transcriptional activity of COX-2 promoter in the presence of hypoxia treatment (Fig. 5A). To confirm the level of COX-2 expression along with siRNA directed against CDX1, RT–PCR and western blotting were performed in hypoxia-treated cells. Knock-down of CDX1 leads to reduction of COX-2 mRNA and protein level (Fig. 5B). These results demonstrate that CDX1 is involved in the hypoxia-induced COX-2 expression. Thus, hypoxia induces COX-2 expression in ovarian cells by inducing CDX1 expression through transcriptional regulation of SHP. In order to further confirm the positive correlation of CDX1 expression together with COX-2 induction in vivo, we used human primary endometrial cells, including ES, ORS...
and OBS cells, to evaluate protein expression by using western blotting. Consistent with in vitro experiments, CDX1 (not CDX2) was increased its protein expression in ORS and OBS compared with ES cells together with COX-2 induction (Fig. 5C). These results indicate that CDX1 regulated by SHP activation is required to induce COX-2 expression in endometriosis.

Discussion
Endometriosis is susceptible to neoplasia induction. Understanding the steps leading to endometriosis is crucial for the identification of early prognostic or diagnostic markers of tumor development. Chronic inflammation and associated endometriosis are fairly common precancerous conditions for ovarian cancer (Sano et al., 1994; Olivares et al., 2008).

The detailed distribution of CDX1 and 2 in a variety of tumors has been reported previously. The previously reported high rates of CDX-2 immunoreactivity in endometrioid and especially in mucinous ovarian carcinomas might in part be explained by a misclassification of metastases as ovarian primaries. Although CDX-2 appears to be an excellent marker for metastases to the ovaries from intestinal carcinomas, it should be noted that poorly differentiated intestinal carcinomas may lose their CDX-2 expression and that rare primary ovarian tumors may be focally positive for CDX-2, conceivably as the result of a form of intestinal differentiation.

Several studies have previously reported that the expression of CDX1 and CDX2 plays a pivotal role in the development of intestinal metaplasia, and directs programs to induce intestinal metaplasia with aberrant differentiation, structures and proliferation activities. Until now there is no information on CDX1 induction in endometrial cells. In this study, we have shown that hypoxia increases CDX1 expression through a cascade transcriptional activation of HIF1α and SHP (Fig. 4). Interestingly, CDX1, but not CDX2, exerts a significant effect on the COX-2 promoter in A2780 cells, although the two genes harbor the same CDX-binding sites (TTTAT). These observations indicate that CDX1 may co-operate with other transcription factors, including SHP, and that these factors are involved in the regulation of COX-2 expression. Even though the first finding of hypoxia-induced
expression of COX-2, SHP and CDX1 in endometriosis tissue cells, we performed most of the assays on two different human ovarian cell lines (A2780 and SKOV3 cells). For our experiments, primary culture human endometriotic cells were available for examining the effect of hypoxia on specific gene induction, but the transfection efficiency of the cells was very low to obtain a significant transactivation and gene expression of target genes. In order to overcome this problem, we performed the transfection experiments of reporter constructs and mammalian expression plasmids by using two different human ovarian cell lines instead of primary cells from endometriosis tissue.

Hypoxia is an early phenomenon in the development of transplanted lesions and is a strong stimulus for the growth of new blood vessels under the regulation of the HIF1α-vascular endothelial growth factor pathway. Generally, under low oxygen levels, the transcription factor HIF-1α is not hydroxylated, avoiding binding to the von-Hippel–Lindau protein and thereby inhibiting its ubiquitination and degradation. HIF-1α then translocates into the nucleus, forms a complex with HIF-1α/aryl hydrocarbon nuclear receptor translocator, and binds to HREs on various genes (Wang et al., 1995). There is evidence that the relationship between HIF-1α activation and inflammation appears to be bidirectional since numerous studies have shown that HIF-1α is a central regulator of inflammation and innate immunity (Nizet and Johnson, 2009), while at the same time proinflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1β, nitric oxide and lipopolysaccharide have been shown to induce HIF-1α expression in cells even under normoxic conditions (Koury et al., 2004). In serial results (Figs 3–5), our findings suggest that the hypoxia increases COX-2 expression, which is a major regulator of inflammatory responses, through transactivation cascade reaction of HIF1α, SHP and CDX1 transcriptional factors in human ovary cells.

The COX-2 is also up-regulated by progesterone withdrawal. Subsequent increased synthesis of PGE2 and PGs F2α (PGF2α) in the endometrium has important implications for menstruation. There are significant numbers of PGs receptors in the endometrium, particularly surrounding the vasculature. PGE2 is a potent vasodilator, leading to increased edema and may contribute to pain at the time of menstruation. Administration of PGE2 to healthy participants increased the diameter of cranial vessels and resulted in headache, demonstrating the involvement of this prostanoid in the sensation of pain. Interestingly, PGE2 has been shown to act synergistically with IL-8 (CXCL8)
to increase plasma leakage and neutrophil accumulation in the skin; PGF2α induces myometrial contractions and vasoconstriction. It is postulated that a period of transient hypoxia may occur in the uppermost endometrial zones as the spiral arterioles constrict following progesterone withdrawal.

COX-2 induction is highly dependent on change of intracellular hormone milieu in endometrial cells. Endometriosis is an estrogen-dependent disease associated with enhanced aromatase expression and local estrogen production in endometriotic tissues (Bulun et al., 2001). Although retrograde menstruation with subsequent implantation and growth of endometrial cells within the peritoneal cavity is a widely accepted mechanism, multiple lines of evidence suggest that inflammation plays a critical role in the pathogenesis of this disease (Lebovic et al., 2001). Estrogen synthesis from C19 steroids is catalyzed by aromatase P450, product of the aromatase/CYP19 gene. Aromatase expression in endometriosis cells is induced via the COX-2-PGE2 pathway, resulting in increased cAMP formation (Bulun et al., 2005). Furthermore, the finding that E2-17β stimulates COX-2 expression suggests that E2 and COX-2 exist in a positive feedback loop (Tamura et al., 2004). E2 plays an important role in controlling the expression of genes involved in a wide variety of biological, inflammatory and neoplastic processes (Nilsson et al., 2001). Our results may give an explanation that hypoxia increases the COX-2-PGE2 production, possibly inducing aromatase expression, and in subsequent estrogen production and an increase of endometriosis.

Here, we identified that hypoxia increases SHP induction by HIF1α transactivation. SHP also acts as a coactivator for NF-kB in inflammatory induction. The result of Fig. 1 shows NF-kB induction together with HIF1α and SHP in endometrial cells by hypoxia treatment. NF-kB is an inducible transcription factor known to increase the expression of a variety of inflammatory molecules. Human endometrial biopsies have been shown to express components of the NF-kB pathway and there is evidence for the activation of NF-kB during the perimenstrual phase. Progesterone has inhibitory effects on NF-kB activity, mediated by increasing IkB production or by competing with NF-kB for recognition sites on relevant genes (Lousse et al., 2009). In this way, progesterone modulates the endometrial inflammatory environment by suppressing NF-kB activity until menstruation is required. The influence of estrogen on endometrial NF-kB activity is less clear, with stimulatory or inhibitory actions observed depending on the cell type studied. Hypoxic conditions have also been shown to stimulate NF-kB signaling and this may modulate the inflammatory response at the time of menstruation. Although primarily a regulator of inflammatory gene expression, NF-kB also inhibits apoptosis and may enable cell survival during the hypoxic insult in the perimenstrual phase.

In conclusion, this study demonstrates the transcriptional mechanisms by which hypoxia induces COX-2 expression in endometriosis development. Specifically, the results of this study demonstrate that hypoxia activates HIF1α followed by SHP induction, which induces the expression of CDX1 in endometrial cells. In turn, CDX1 is capable of inducing COX-2. Moreover, the present observations suggest that the identification of the factors required for the hypoxia-induced inflammation in the process of endometriosis and the clarification of the molecular mechanisms responsible for an early phenotypic change in endometriosis may facilitate the development of therapeutic interventions.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

**Authors’ roles**

K.H.K. carried out experiments and wrote the manuscript. H.Y.K. conducted experiments and interpreted the data. H.H.K. was involved in the acquisition of experimental materials and cells and interpreted the data. K.S.L. took part in experimental design and received research fund. J.C. played role in experimental design and wrote the manuscript.

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**References**


