

Dienogest reduces HSD17β1 expression and activity in endometriosis

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Abstract

Endometriosis is an estrogen-dependent disease. Abnormally biosynthesized estrogens in endometriotic tissues induce the growth of the lesion and worsen endometriosis-associated pelvic pain. Dienogest (DNG), a selective progesterone receptor agonist, is widely used to treat endometriosis and efficiently relieves the symptoms. However, its pharmacological action remains unknown. In this study, we elucidated the effect of DNG on enzymes involved in local estrogen metabolism in endometriosis. Surgically obtained specimens of 23 ovarian endometriomas (OE) and their homologous endometrium (EE), ten OE treated with DNG (OE w/D), and 19 normal endometria without endometriosis (NE) were analyzed. Spheroid cultures of stromal cells (SCs) were treated with DNG and progesterone. The expression of aromatase, 17β-hydroxysteroid dehydrogenase 1 (HSD17β1), HSD17β2, HSD17β7, HSD17β12, steroid sulfatase (STS), and estrogen sulfotransferase (EST) was evaluated by real-time quantitative PCR. The activity and protein level of HSD17β1 were measured with an enzyme assay using radiolabeled estrogens and immunohistochemistry respectively. OESCs showed increased expression of aromatase, HSD17β1, STS, and EST, along with decreased HSD17β2 expression, when compared with stromal cells from normal endometria without endometriosis (NESCs) ($P < 0.01$) or stromal cells from homologous endometrium (EESCs) ($P < 0.01$). In OESCs, DNG inhibited HSD17β1 expression and enzyme activity at 10^{-7} M ($P < 0.01$). Results of immunohistochemical analysis displayed reduced HSD17β1 staining intensity in OE w/D ($P < 0.05$). In conclusion, DNG exerts comprehensive inhibition of abnormal estrogen production through inhibition of aromatase and HSD17β1, contributing to a therapeutic effect of DNG on endometriosis.

Key Words

- ▶ 17β-hydroxysteroid dehydrogenase 1
- ▶ dienogest
- ▶ endometriosis
- ▶ ovarian endometrioma
- ▶ spheroid culture

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Introduction

Endometriosis is defined as the presence of endometrium-like tissues at extra-uterine sites. Clinical symptoms associated with endometriosis include pelvic pain, dysmenorrhea, dyspareunia, and infertility (Giudice 2010). There is marked relief of symptoms after menopause, clearly demonstrating the dependency of endometriosis on estrogens. Besides systemic circulating estrogens

secreted from the ovaries, abnormally biosynthesized estrogens in endometriotic tissues also contribute to the growth of the lesion and worsening symptoms (Bulun 2009). In the eutopic and ectopic endometria of women with endometriosis, overexpressed aromatase (also known as estrogen synthase) biosynthesizes estrogens, namely estrone and estradiol, from the androgens,

androstenedione, and testosterone respectively (Noble *et al.* 1996, Kitawaki *et al.* 1997, Matsuzaki *et al.* 2006, Dassen *et al.* 2007, Smuc *et al.* 2007). In these tissues, estradiol, the most potent estrogen, is predominantly synthesized from less potent estrone by 17β-hydroxysteroid dehydrogenase 1 (HSD17β1), and the reverse reaction is catalyzed mainly by HSD17β2. In endometriotic tissues, the expression of HSD17β1 is higher than that of HSD17β2; thus, the reaction is tilted in favor of producing estradiol (Zeitoun *et al.* 1998, Dassen *et al.* 2007). The other major source of estrogens is estrone sulfate, an inactive conjugated form abundant in the circulation. Estrone sulfate is desulfated to estrone by steroid sulfatase (STS) and estrone is inactivated by estrogen sulfotransferase (EST) (Utsunomiya *et al.* 2004, Colette *et al.* 2013). Understanding how the aberrant expression of these enzymes in endometriosis contributes to local estrogen production and metabolism will allow the development of improved therapeutic agents.

Dienogest (DNG), a selective progesterone (P₄) receptor (PR) agonist, is widely used to treat endometriosis (McCormack 2010) and efficiently relieves endometriosis-associated pelvic pain (Harada *et al.* 2009, Momoeda *et al.* 2009, Strowitzki *et al.* 2010, Petraglia *et al.* 2012). DNG directly inhibits PR-mediated cell proliferation (Okada *et al.* 2001, Fu *et al.* 2008, Shimizu *et al.* 2009) and production of the inflammatory factors involved in the pathology of endometriosis, such as prostaglandin estradiol (E₂) (Shimizu *et al.* 2011, Yamanaka *et al.* 2012), inflammatory cytokines (Horie *et al.* 2005), Toll-like receptor 4 (Mita *et al.* 2011), and nerve growth factor (Mita *et al.* 2014). Suppression of these inflammatory factors is considered to contribute, in part, to the improvement of pain symptoms. DNG restores the antigen-presenting ability of peritoneal fluid macrophages by increasing human leukocyte antigen-DR expression (Maeda *et al.* 2014). DNG also suppresses aromatase

expression in human immortalized endometrial epithelial cells (Shimizu *et al.* 2011) and primary cultured stromal cells (SCs) derived from ovarian endometrioma (OE) (Yamanaka *et al.* 2012). However, the effect of DNG on other estrogen-metabolizing enzymes in endometriotic cells remains unknown, and a more detailed analysis is needed to understand its clinical effectiveness and pharmacological function.

The purpose of this study was to investigate the effect of DNG on enzymes involved in estrogen metabolism using spheroid cultures of primary cultured SCs derived from OE, endometrium with endometriosis (EE), and normal endometrium without endometriosis (NE).

Patients and methods

Patients and samples

Patient characteristics are given in Table 1. OE tissues from patients (*n*=23) who did not receive any hormonal treatment and their homologous EE specimens (*n*=10), in addition to OE specimens from patients treated with DNG at a dose of 1 mg twice daily for 3–5 months (OE treated with DNG (OE w/D)) (*n*=11), were obtained from women undergoing surgery for OE. NE specimens were obtained from women undergoing surgery for uterine fibroids (*n*=19). All women were of reproductive age, and all specimens, with the exception of OE w/D, were collected at the proliferative phase of the regular menstrual cycle. Women who had undergone hormonal treatments within 6 months before surgery were excluded. OE w/D specimens were not used for *in vitro* experiments to avoid the effect of previous DNG exposure on the results. The endometriosis stages were evaluated according to the American Society for Reproductive Medicine classification of endometriosis. This study was conducted in accordance with the guidelines of the Declaration of

Table 1 Clinical characteristics of study patients. Values are presented as means ± s.e.m.

	NE (<i>n</i> =19)	EE (<i>n</i> =10)	OE (<i>n</i> =23)	OE w/D (<i>n</i> =11)
Age (years)	41.8 ± 4.3	41.4 ± 3.9	32.5 ± 7.0* [†]	37.5 ± 4.2
CA-125 (U/ml)	NA	68.9 ± 29.1	82.5 ± 83.4	68.0 ± 55.7
r-ASRM stage (%)				
III	NA	6 (60)	14 (61)	6 (55)
IV	NA	4 (40)	9 (39)	5 (45)
Duration of drug administration (weeks)	NA	NA	NA	13.4 ± 6.0

P values were obtained by Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure or χ^2 test. NE, normal endometrium; EE, endometrium with endometriosis; OE, ovarian endometrioma; OE w/D, OE treated with dienogest; r-ASRM, revised American Society for Reproductive Medicine; DNG, dienogest. **P*<0.01 versus NE and [†]*P*<0.05 versus EE.

Helsinki and was approved by the institutional review board of the Kyoto Prefectural University of Medicine. Informed consent was obtained from all patients.

Isolation and culture of SCs

The isolation and culture of SCs was conducted as described previously (Yamanaka *et al.* 2012). Briefly, tissue digestion was performed with 2.5% collagenase (Nacalai Tesque, Kyoto, Japan) and 15 IU/ml of DNase I (Takara Shuzo, Tokyo, Japan). After filtering through a nylon cell strainer, the digested cells were centrifuged in lymphocyte separation solution (Nacalai Tesque) to remove the red blood cells. The >95% purity of SC preparations was confirmed by positive staining for CD10 and vimentin and negative staining for cytokeratin, CD31, and CD45. The cells were cultured in DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (100 µg/ml), under a humidified atmosphere at 37 °C in 5% CO₂. The cells that reached subconfluence were dispersed using 0.1% trypsin (Nacalai Tesque) and resuspended in phenol-red-free DMEM/Ham's F-12 (Nacalai Tesque) supplemented with 10% dextran-coated charcoal-treated FBS and 1% penicillin and streptomycin (100 µg/ml). For mRNA analysis, SCs were subcultured in U-bottom 96-well culture plates (Sumilon) at a density of 4 × 10⁴ cells/well to form spheroids. For HSD17β1 activity assays, SCs were plated into six-well culture plates at a density of 4 × 10⁵ cells/well to form monolayers. The OESC spheroid expression of estrogen receptor α (ERα (ESR1)), ERβ (ESR2), PR, aromatase, cyclooxygenase-2 (COX2), and nuclear factor-κ B (NFκB) p50 subunit nuclear localization was validated by immunocytochemistry as described previously (Yamanaka *et al.* 2012).

Treatment of cultured SCs with DNG or P₄

The culture medium was replaced either after 72 h (spheroid culture for RNA extraction) or when cells reached subconfluence (HSD17β1 activity assay) by medium with or without DNG (10⁻⁸, 10⁻⁷, and 10⁻⁶ M; Bayer Schering Pharma, Berlin, Germany) or P₄ (10⁻⁸, 10⁻⁷, and 10⁻⁶ M; Sigma-Aldrich), and the cells were incubated for a further 48 h.

RNA extraction, cDNA preparation, and real-time PCR

Total RNA was extracted from cultured SCs using the RNeasy Mini Kit (Qiagen). After quantification and

determination of the quality of the RNA by u.v. absorption (OD 260 nm/280 nm) using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA, USA), cDNA was synthesized using the SuperScript III first-strand synthesis system (Invitrogen) and a GeneAmp PCR 9700 machine (Applied Biosystems). Quantitative real-time PCR was conducted using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) and a StepOne Real-Time PCR System (Applied Biosystems) with TaqMan assay primer/probe sets (Applied Biosystems) for the target genes: aromatase (*CYP19A1*) (Hs00240671_m1), *HSD17β1* (Hs00166219_g1), *HSD17β2* (Hs00157993_m1), *HSD17β7* (Hs00367686_m1), *HSD17β12* (Hs00275054_m1), *STS* (Hs00996676_m1), *EST (SULT1E1)* (Hs00960941_m1), and endogenous control *GAPDH* (Hs03929097_g1). Real-time quantitative PCR was performed under the following thermal cycling conditions: denaturing at 95 °C for 60 s; 3 s at 95 °C; 40 cycles of 30 s at 60 °C. Threshold cycle (Ct) values were calculated using the ΔΔCt method.

HSD17β1 enzyme assays

HSD17β1 activity was measured using thin layer chromatography, as described previously (Kitawaki *et al.* 2000). Briefly, cells were washed twice with phenol-red-free DMEM/Ham's F-12, and then incubated at 37 °C/5% CO₂ for 6 h with 0.5 ml of serum-free medium containing [6,7-³H]estrone (Perkin Elmer, Waltham, MA, USA) (1.8 × 10⁶ dpm, 37 µM). The reaction was stopped by transferring the medium to the test tubes containing 2 ml chloroform and the corresponding carrier steroids: [4-¹⁴C]estradiol (Perkin Elmer) (1.3 × 10⁴ dpm) and non-radioactive estrone and estradiol (0.2 mg each). The steroids were isolated by thin-layer chromatography using Silicagel 60 F254 (0.25 mm; Merck) in a system of chloroform:ethyl acetate (4:1, v/v). The aliquot was mixed with Clear-sol I (Nacalai Tesque), and radioactivity was measured using a scintillation counter (Beckman Coulter, Fullerton, CA, USA). Enzyme activity was calculated and normalized according to the ratios of the estradiol formed. Protein concentration (pmol/mg protein per h) was measured by the Bradford method.

Immunohistochemistry

Specimens from OE, OE w/D, EE, and NE were stained immunohistochemically as described previously (Yamanaka *et al.* 2012) using an anti-HSD17β1 antibody (200 µg/ml; Abcam, Cambridge, UK). Normal term placenta tissue was used as a positive control. Because the cell components

of epithelial cells and SCs were considerably different between eutopic endometrium and OE, we compared the immunostaining intensity in SCs using the *H*-score, a semi-quantitative index involving an algorithm described previously (Yamanaka *et al.* 2012). Briefly, two independent observers evaluated approximately 500 cells/slide and scored them as follows: 3×percentage of strongly staining cells+2×percentage of moderately staining cells+percentage of weakly staining cells. The *H*-score was calculated as the mean of the two scores.

Statistical analyses

The mRNA expression levels of enzymes in the three types of SCs were analyzed by Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure because of the unequal variances in the results. Results of real-time PCR and the HSD17β1 activity assay measuring the drugs' effects on mRNA expression and enzyme activity levels in OESCs were assessed by repeated measures ANOVA followed by multiple comparisons using Dunnett's procedure. Statistical analysis of the immunohistochemical results was performed using an

unpaired *t*-test. Each assay for individual experiments was performed in triplicate. Data are presented as means±s.e.m. *P* values of <0.05 were considered statistically significant.

Results

mRNA expression of enzymes in spheroid-cultured SCs

In OESCs, the mRNA expression levels of aromatase ($P<0.01$), *HSD17β1* ($P<0.01$), *STS* ($P<0.01$), and *EST* ($P<0.01$) were greater compared with those in NESCs and EESCs. In NESCs or EESCs, we detected neither aromatase nor *EST* mRNA expression and an extremely low level of *HSD17β1* mRNA expression. In contrast, *HSD17β2* mRNA expression was lower in OESCs compared with that in NESCs ($P<0.01$) and EESCs ($P<0.01$) (Fig. 1A).

Effects of drugs on enzymes in OESCs

Incubating the spheroids for 48 h with DNG (10^{-7} M ($P<0.01$) and 10^{-6} M ($P<0.01$)) and P_4 (10^{-7} M ($P<0.05$) and 10^{-6} M ($P<0.01$)) significantly decreased

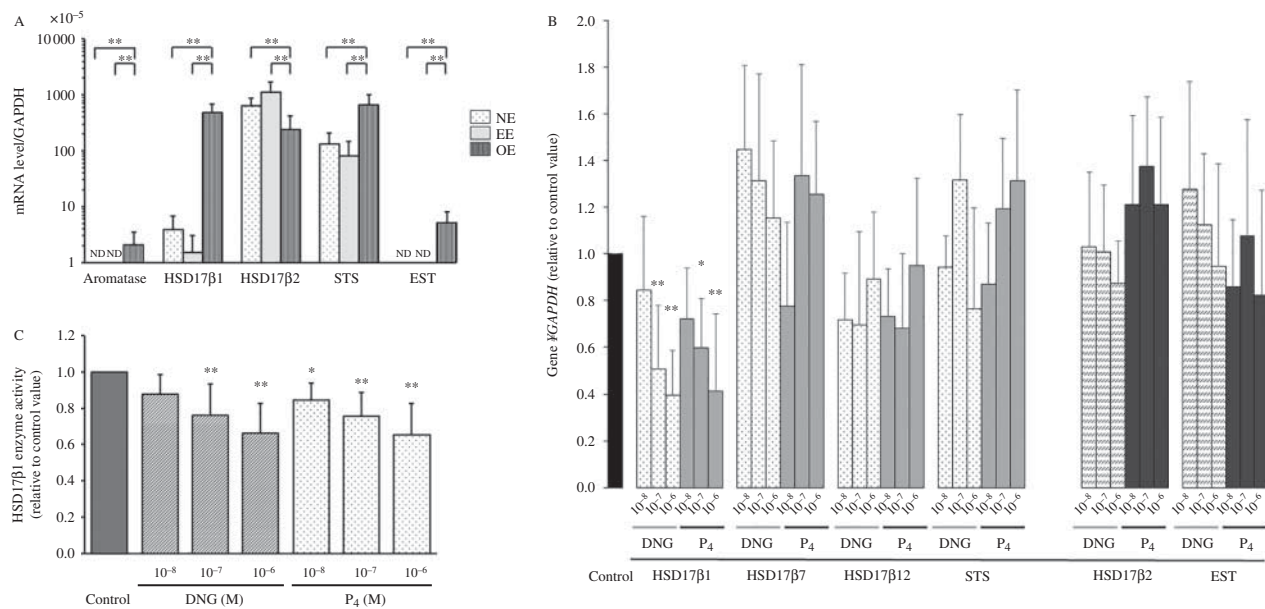
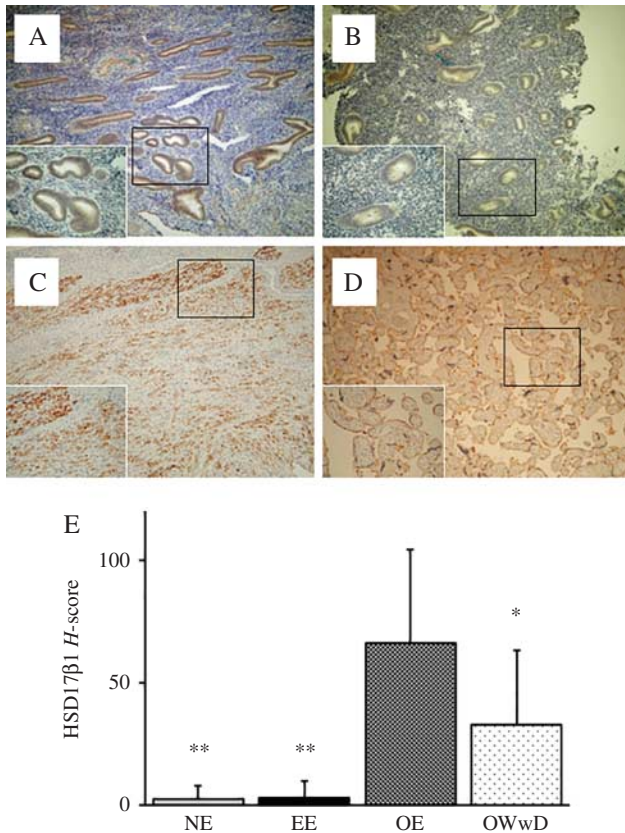


Figure 1

(A) The baseline mRNA expression levels of estrogen-metabolizing enzymes in spheroid-cultured stromal cells (SCs) derived from normal endometrium (NE), endometrium with endometriosis (EE), and OE. (B) Effects of dienogest (DNG) and progesterone (P_4) on mRNA expression levels of enzymes in OESCs (at least seven separate experiments respectively) and on (C) 17β-hydroxysteroid dehydrogenase 1 (HSD17β1) activity in OESCs ($n=8$). All assays were performed in triplicate, and data

are presented as means±s.e.m. *P* values for the statistical analysis of the mRNA expression of enzymes in SCs in spheroid culture are based on Kruskal–Wallis ANOVA followed by multiple comparisons using Scheffe's procedure, and effects of drugs on OESC enzyme mRNA expression and activity are based on repeated measures ANOVA followed by multiple comparisons using Dunnett's procedure. ND, not detectable; * $P<0.05$ and ** $P<0.01$ versus control.

**Figure 2**

Representative immunohistochemical staining of 17 β -hydroxysteroid dehydrogenase 1 (HSD17 β 1) in (A) normal endometrium (NE), (B) endometriosis with endometriosis (EE), (C) ovarian endometrioma (OE), and (D) normal term placenta as a positive control. (E) The immunostaining intensity of NE ($n=19$), EE ($n=10$), OE ($n=23$), and OE treated with dienogest (OE w/D) ($n=11$) was scored with semi-quantitative index H-scores. Areas shown at a higher magnification are indicated by rectangles. Data are presented as means \pm S.E.M. P values are based on an unpaired t -test. * $P<0.05$ and ** $P<0.01$ versus OE.

the expression of *HSD17 β 1* mRNA compared with the controls. However, DNG or P_4 did not significantly suppress *HSD17 β 2*, *HSD17 β 7*, *HSD17 β 12*, *STS*, or *EST* mRNA expression (Fig. 1B). In parallel with the mRNA results, DNG (10^{-7} M ($P<0.01$) and 10^{-6} M ($P<0.01$)) and P_4 (10^{-8} M ($P<0.05$), 10^{-7} M ($P<0.01$), and 10^{-6} M ($P<0.01$)) significantly suppressed the catalytic activity of HSD17 β 1 (Fig. 1C).

Immunohistochemistry

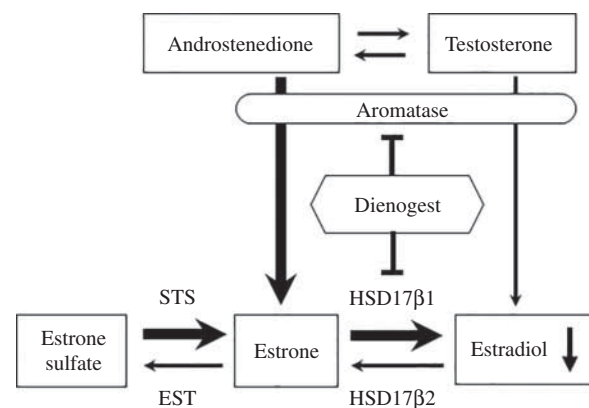
Immunohistochemical analysis showed HSD17 β 1 expression in the cytoplasm of epithelial cells and SCs of NE (Fig. 2A), EE (Fig. 2B), and OE (Fig. 2C). The immunostaining intensity of HSD17 β 1 was greater in OESCs when compared with NE ($P<0.01$) and EE

($P<0.01$). Moreover, there was a significant reduction in the immunostaining intensity in the OE w/D group ($P<0.05$) (Fig. 2E).

Discussion

In this study, we demonstrated DNG-mediated inhibition of mRNA expression, catalytic activity, and protein expression of HSD17 β 1 in endometriosis. Taken together with the previous findings that DNG inhibits aromatase in endometriosis (Shimizu *et al.* 2011, Yamanaka *et al.* 2012), DNG exerts comprehensive inhibition of abnormal estrogen production by the inhibition of two key enzymes that regulate estradiol production (Fig. 3). These actions of DNG contribute, in part, to its therapeutic effect on endometriosis.

In order to comprehensively examine the expression patterns of estrogen-metabolizing enzymes in OESCs compared with those in EESCs or NESCs, we employed a three-dimensional spheroid culture system characterized by multicellular aggregates of cells and extracellular matrices. This culture system produces sufficient baseline levels of proinflammatory factors (Enzerink *et al.* 2009, Vaheri *et al.* 2009), and spheroids of human immortalized endometrial epithelial cells (Shimizu *et al.* 2011) and primary cultured OESCs (Yamanaka *et al.* 2012) express higher levels of aromatase, COX2, and prostaglandin E_2 compared with the corresponding monolayer cultures. The results of this study indicated that HSD17 β 1 localizes in the cytoplasm of both epithelial cells and SCs. In OE, however, the majority of cells are SCs. Thus, the OESC

**Figure 3**

Scheme summarizing dienogest inhibition of estrogen production in endometriosis. Dienogest inhibits both aromatase and 17 β -hydroxysteroid dehydrogenase 1 (HSD17 β 1), the key enzymes in estradiol biosynthesis. STS, steroid sulfatase; EST, estrogen sulfotransferase; HSD17 β 2, 17 β -hydroxysteroid dehydrogenase 2.

spheroids are considered to mimic the local environment of enzyme expression.

In the spheroid-cultured OESCs, we detected very low level of aromatase mRNA expression, whereas it was not detectable in EESCs or NESCs. This is in agreement with results from earlier studies, in which aromatase is only detectable in studies using immunohistochemistry and those using homogenized specimens just after sampling but not in monolayer cultured cells (Kitawaki *et al.* 1997, Dassen *et al.* 2007, Smuc *et al.* 2007). In OESCs, we detected overexpression of HSD17β1 mRNA and protein levels, responsible for activating estrogenic potency, whereas very low levels of mRNA and protein expression were observed in EESCs and NESCs. In contrast, the mRNA expression of HSD17β2, responsible for weakening estrogenic potency, was significantly lower in OESCs compared with EESCs and NESCs. This balance between the expressions of the two enzymes indicates that estradiol is more likely to be produced in OESCs compared with eutopic endometrium, which is concordant with results from previous studies (Zeitoun *et al.* 1998, Matsuzaki *et al.* 2006).

Furthermore, the expression of STS, also responsible for activating estrogenic potency, was significantly higher in OESCs compared with EESCs and NESCs, which is also consistent with previous findings (Utsunomiya *et al.* 2004, Colette *et al.* 2013). Dassen *et al.* (2007) reported high STS mRNA expression in both eutopic and ectopic endometrium, but no difference between the two tissues.

In contrast to the high levels of expression of STS, we detected very low levels of mRNA expression of *EST*, responsible for inactivating estrone in OESCs, whereas it was not detectable in EESCs or NESCs. Colette *et al.* (2013) reported very low levels of *EST* mRNA expression in both eutopic and ectopic endometrium, but found no difference between the two tissues. Utsunomiya *et al.* (2004) showed that *EST* was expressed in the endometrium but only during the secretory phase. In this study, we obtained specimens during the proliferative phase to eliminate the effect of P₄. This balance of the STS and *EST* expression indicates that estrone is more favorably produced in OESCs as well as EESCs and NESCs during the proliferative phase. The significance of the differences in expression of STS and *EST* between these cells remains to be elucidated.

Using this experimental model, it was determined that DNG significantly inhibited *HSD17β1* mRNA expression and its enzyme activity at 10⁻⁷ M in OESCs. These concentrations are equivalent to the blood level of mice administered 1 mg of DNG twice daily and patients administered 2 mg of DNG daily (Meriggiola *et al.* 2002, Sasagawa *et al.* 2008). The *in vitro* inhibitory effects of DNG

are supported by the *in vivo* data from this study, demonstrating that DNG treatment for 3–5 months resulted in decreased HSD17β1 protein expression in OE.

The conversion of estrone to estradiol is also mediated through HSD17β7 and HSDβ12 as well as HSDβ1 (Moeller & Adamski 2006). DNG inhibited only *HSD17β1* but not *17HSDβ7* or *12* mRNA expression, which indicates that DNG reduces local estrogen production by the suppression of HSD17β1 in human OESCs.

We and other researchers have shown that the PR is involved in the mechanism of DNG-inhibited cell proliferation (Okada *et al.* 2001, Shimizu *et al.* 2009) and the expression of inflammatory factors (Mita *et al.* 2011), nerve growth factor, (Mita *et al.* 2014), and aromatase (Yamanaka *et al.* 2012). Although the *HSD17β1* gene lacks a P₄-responsive element in its promoter region, progestins including DNG downregulated *HSD17β1* and upregulated *HSD17β2* expression in immortalized endometriotic epithelial cells (Beranic & Rizner 2012). DNG inhibits the DNA-binding activity of NFκB, a key regulator of various pathological and inflammatory responses in endometriosis such as interleukin-8 production in human OESCs (Horie *et al.* 2005, Shimizu *et al.* 2011, Yamanaka *et al.* 2012). Bulun proposed a vicious cycle of an estrogen-dependent mechanism of endometriosis growth (Bulun 2009). Estradiol produced locally by aromatase stimulates tissue growth of endometriosis and upregulates COX2 via ERβ activation. COX2 overexpression results in an excess of prostaglandin E₂, which further stimulates aromatase expression via the orphan nuclear receptor steroidogenic factor 1. Furthermore, ERβ activation downregulates PR, which leads to reduced induction of HSD17β2 via retinoic acid (Zeitoun *et al.* 1998). The combination of upregulation of aromatase and downregulation of HSD17β2 contributes to the abnormally high levels of estradiol in endometriotic tissue. In addition to inhibition of aromatase, DNG inhibits HSD17β1, resulting in further reductions in local estradiol concentration. This interrupts the vicious cycle of endometriosis growth and also relieves endometriosis-associated pelvic pain by inhibiting prostaglandin E₂ production. The effect of inhibiting HSD17β1 on endometriosis has been demonstrated (Delvoux *et al.* 2014) and several HSD17β1 inhibitors have been developed and used in preclinical studies (Day *et al.* 2008, Poirier 2011). DNG is widely used in clinics to treat endometriosis with fewer side effects. We believe that the identification of the molecular mechanisms behind the therapeutic effect of DNG described here will lead to better understanding of the pathophysiology of endometriosis.

Declaration of interest

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

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