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Deoxyribonucleic acid methyltransferases and methyl-CpG-binding domain proteins in human endometrium and endometriosis

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Objective: To determine [1] expression levels of both DNA methyltransferases (DNMTs) and methyl-CpG-binding domain proteins (MBDs) in human endometrium throughout the menstrual cycle and in eutopic and ectopic endometrium of patients with endometriosis and [2] hormone responsiveness of DNMT and MBD expression in explant cultures of proliferative phase endometrium.
Design: In vitro study.
Setting: Academic medical center.
Patient(s): Premenopausal women with and without endometriosis.
Intervention(s): Explant cultures of proliferative phase endometrium were treated with vehicle, 17β-E_2, or a combination of E_2 and P (E_2 + P) for 24 hours.
Main Outcome Measure(s): Expression levels of DNMT1, DNMT2, and DNMT3B and MBD1, MBD2, and MeCP2 with use of real-time quantitative polymerase chain reaction.
Result(s): Expression levels of DNMT1 and MBD2 were significantly higher in secretory-phase endometrium compared with proliferative endometrium and menstrual endometrium. In explant cultures, treatment with E_2 + P resulted in significant up-regulation of DNMT1 and MBD2. Expression levels of several DNMTs and MBDs were significantly lower in endometriotic lesions compared with eutopic endometrium of women with endometriosis and disease-free controls.
Conclusion(s): These findings suggest a role for DNMTs and MBDs in the growth and differentiation of the human endometrium and support the notion that endometriosis may be an epigenetic disease. (Fertil Steril® 2011;95: 1421–7.)
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Key Words: Endometriosis, endometrium, DNA methylation, epigenetics, DNMT, MBD

Endometriosis is a benign gynecologic disorder that is characterized by the presence of endometrial glands and stroma at ectopic sites outside the uterine cavity. Its exact pathogenesis remains enigmatic. Recent reports support a role for epigenetic processes in the pathogenesis of endometriosis (1–4). Epigenetics refers to the study of heritable changes in gene expression that occur *without* changes in the underlying DNA sequence (5).

The most studied epigenetic alteration is DNA methylation, which refers to the addition of a methyl group to the 5′ position of cytosines that precede a guanine in the DNA sequence, the CpG dinucleotide. Unmethylated CpGs are clustered in CpG islands and often are associated with transcriptional start sites in promoter regions. Nowadays it is widely recognized that promoter hypermethylation is associated with condensed chromatin and silencing of gene expression.

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The mammalian DNA methylation machinery is composed of two components, DNA methyltransferases (DNMTs) and methyl-CpG-binding domain proteins (MBDs). Deoxyribonucleic acid methyltransferases are enzymes that catalyze the addition of methyl groups to CpG dinucleotides. DNMT1 is regarded as the major enzyme responsible for maintaining existing DNA methylation patterns during replication. DNMT3A and DNMT3B are thought of as de novo methyltransferases that are involved in the postreplicative methylation of previously unmethylated DNA. Finally, DNMT2 is an enzyme with strong sequence similarities with 5-methylcytosine methyltransferases of both prokaryotes and eukaryotes but was shown not to methylate DNA (6).

The crosstalk between DNA methylation and histone modifications is established by a family of proteins that contain a methyl-CpG binding domain, commonly known as MBD proteins. On the basis of several studies (7, 8), a model has been proposed for DNA methylation–mediated transcriptional silencing. According to this model, MBDs specifically recognize and subsequently bind to methylated CpGs and recruit histone deacetylases (HDACs). The HDAC removes acetyl groups from the lysines on histone tails, resulting in chromatin condensation and transcriptional inactivation. The process of gene silencing therefore requires the combined action of both DNMTs and MBDs. To date, five MBDs have been cloned: MeCP2, MBD1, MBD2, MBD3, and MBD4. Of these,

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MeCP2, MBD1, and MBD2 have been shown to function as transcriptional repressors (9).

A significant correlation was demonstrated between DNA methylation and the expression of progesterone receptor in normal endometrium (10), suggesting that DNA methylation may be involved in steroid-hormone–dependent endometrial growth and differentiation. In addition, overexpression of steroidogenic factor 1 and estrogen receptor β in endometriotic tissue was shown to be the result of demethylation of the gene promoters (1, 2).

To date, two studies addressing DNMT expression in endometrium (11) and endometriosis (12) have been performed. However, these studies have focused on DNMT expression solely, and, so far, no studies reporting MBD expression in these tissues are known. Therefore, the aim of this study was to investigate the expression levels of both DNMTs and MBDs in [1] normal endometrium throughout the menstrual cycle and [2] eutopic and ectopic endometrium of women with endometriosis. A second aim was to investigate hormone responsiveness of DNMT and MBD expression. The presence of DNMT and MBD proteins in these tissues was assessed by immunohistochemistry.

MATERIALS AND METHODS

Tissues

As described in more detail later, tissues were collected in two different clinics, the Maastricht University Medical Center, in Maastricht, the Netherlands, and the Leuven Fertility Clinic in Leuven, Belgium.

Tissues collected in the Maastricht University Medical Center Endometrial tissue was collected from 23 women with regular menstrual cycles who underwent surgery for benign indications other than endometriosis in the Maastricht University Medical Center. The tissue was collected from hysterectomy specimens or by Pipelle biopsies during laparoscopy (Pipelle catheter; Unimar Inc., Prodimed, Neuilly-en-Thelle, France). The women were documented not to be taking any kind of steroid medication. All women signed an informed consent, as required by the protocol approved by the Medical Ethical Committee of the Maastricht University Medical Center. Of the 23 biopsy specimens, 3 were collected in the menstrual phase, 8 in the proliferative phase, and another 12 in the secretory phase of the menstrual cycle. Each biopsy sample was divided in three parts: the first part was immediately frozen in liquid nitrogen and stored at -80°C until used for research in this study, and the second part was fixed in 10% buffered formalin for histologic analysis. Of the proliferative phase tissue samples, a third part was kept apart and directly used for endometrial explant cultures (see later). The endometrium was dated according to clinical information with respect to the start of the last menstrual period, which was reconfirmed by histologic examination of the tissue (13).

Explant cultures Part of the proliferative phase endometrium tissue from all 23 collected biopsy specimens was used for explant culture as described earlier (14). The tissue was cultured for 24 hours in the presence of vehicle (0.1% ethanol) or steroid hormones (E_2, 1 nmol/L; P_4, 1 nmol/L; $E_2 + P_4$, 1 nmol/L each). At the end of the experiment, the explants were collected in lysis buffer (SV Total RNA Isolation Kit; Promega, Madison, WI) and stored at -80°C until RNA isolation.

Tissues collected in the Leuven Fertility Clinic Patient material used for the present investigation has been described before (15). In short, tissue was obtained during the proliferative phase of the menstrual cycle from 34 women, including 20 women with laparoscopically confirmed absence of endometriosis and 14 women with both laparoscopically and histologically confirmed stage III to IV endometriosis (American Society for Reproductive Medicine classification [16]). Both eutopic and ectopic tissue was harvested. All patients had a regular menstrual cycle and were documented not to be taking any steroid medication within 6 months before surgery.

Ribonucleic Acid Isolation and Complementary Deoxyribonucleic Acid Synthesis

Total cellular RNA from the cultured explants and uncultured cyclic endometrium was extracted with use of the SV Total RNA Isolation Kit (Promega) according to the manufacturer's protocol. Ribonucleic acid from the tissue samples collected in the Leuven Fertility Clinic was isolated with use of the TRIzol reagent method (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Ribonucleic acid quantity and quality were assessed with a spectrophotometer at 260- and 280-nm wavelengths. For the production of complementary DNA the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used.

Real-time Polymerase Chain Reaction

The TaqMan Gene Expression Assays for DNMT1 (Hs00154749_m1), DNMT2 (Hs00189402_m1), DNMT3B (Hs00171876_m1), MBD1 (Hs00242770_m1), MBD2 (Hs00187506_m1), and MeCP2 (Hs00172845_m1), β-actin (Hs99999903_m1), and cyclophilin A (Hs99999904_m1) were purchased from Applied Biosystems (Foster City, CA). Human cyclophilin A was used as housekeeping gene to normalize the differences in the amount of complementary DNA added to each PCR reaction. To check for the amount of endometrial tissue present in eutopic and ectopic endometrial biopsies, the expression level of 17β-hydroxysteroid dehydrogenase 4 (17βHSD4), a gene previously shown to be expressed at equal levels in eutopic endometrium and endometriotic tissue (15), was measured.

Immunohistochemistry

A summary of the primary and secondary antibodies, antigen retrieval steps, and incubation conditions used is given in Table 1. Antibody binding was visualized with use of the Envision rabbit anti-mouse (for monoclonal antibodies) or anti-goat (for goat polyclonal antibodies) ChemMate Detection Kits (DAKO, Copenhagen, Denmark), followed by incubation with 3, 3′-diaminobenzidine. Negative control slides for the monoclonal antibodies were incubated with mouse IgGs of the same class and same dilution as the primary antibodies. Negative control slides for the goat polyclonal antibodies were incubated with goat IgG at the same dilution as the polyclonal antibodies.

TABLE 1

Primary antibodies and conditions used for immunohistochemical analysis of eutopic endometrium and endometriosis lesions.

Antibody	Species	Catalog no.	IgG class	Dilution	Manufacturer	Incubation	Antigen retrieval
DNMT1	Goat	sc-10222	IgG1	1:750	Santa Cruz Biotechnology, Santa Cruz, CA	2 h room temperature	Tris-EDTA (pH 9.0)
MBD2	Goat	sc-9397	Not specified	1:500	Santa Cruz Biotechnology, Santa Cruz, CA	Overnight 4°C	Tris-EDTA (pH 9.0)
5 MeCyt	Mouse	MCA2201	IgG1	1:100	AbD Serotec, Oxford, United Kingdom	2 h room temperature	Citrate (pH 6.0)

Note: Tris-EDTA = tris(hydroxymethyl)aminomethane–ethylenediaminetetraacetic acid.

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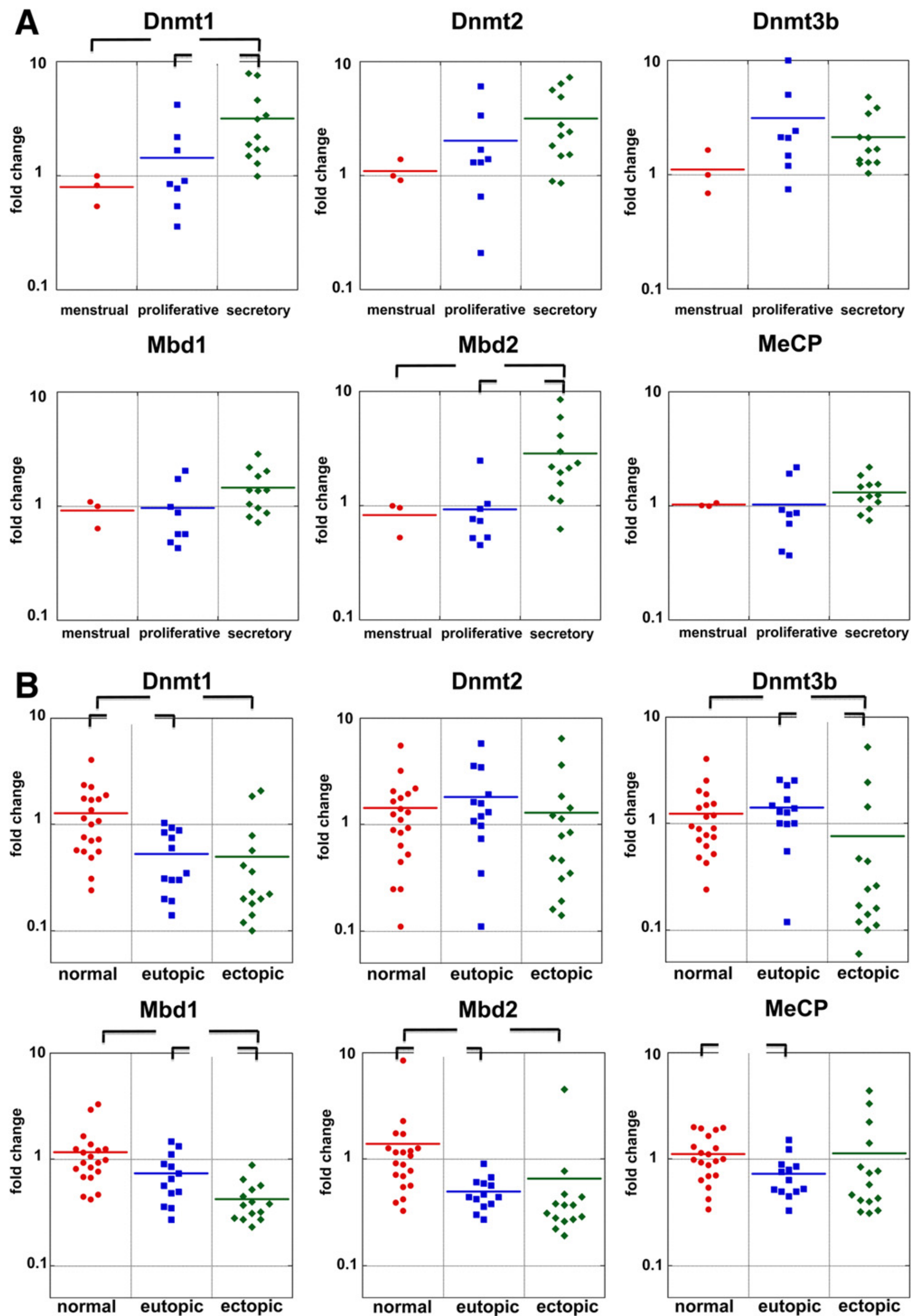
Statistical Tests

Statistical tests were carried out with use of the Statistical Package for the Social Sciences (version 16.0; SPSS Inc., Chicago, IL) statistical analysis package. To evaluate whether expression levels varied significantly throughout the menstrual cycle, the nonparametric unpaired Mann-Whitney *U* test was used to test for differences between these expression levels versus the expression level in the M phase. The nonparametric Wilcoxon signed-rank test was used to test for differences between steroid-treated explants and controls at a confidence level of 95%.

FIGURE 1

Expression levels of DNMT1, DNMT2, DNMT3B, MBD1, MBD2, and MeCP2 in menstrual, proliferative and secretory phase endometrium (**A**) and in eutopic endometrium of controls (*normal*), eutopic endometrium of patients with endometriosis (*eutopic*), and ectopic endometrium of patients with endometriosis (*ectopic*) (**B**).



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RESULTS

Deoxyribonucleic Acid Methyltransferases and Methyl-CpG-binding Domain Protein Expression Throughout the Menstrual Cycle

The relative expression level of DNMT1 was significantly higher in secretory endometrium compared with proliferative (P=.02) and menstrual endometrium (P=.009). Expression levels of DNMT2 and DNMT3B were not significantly different between the cycle phases. Significantly higher expression levels of MBD2 were observed in secretory endometrium compared with proliferative (P=.004) and menstrual (P=.018) endometrium. There were no statistically significant differences in MBD1 and MeCP2 expression throughout the menstrual cycle (Fig. 1A). Immunoreactivity for DNMT1 (Fig. 2A), MBD2 (Fig. 2B), and 5-methylcytosine (Fig. 2C) was observed in nuclei of epithelial and stromal cells of both proliferative and secretory endometrium.

Expression levels of deoxyribonucleic acid methyltransferases and methyl-CpG-binding domain proteins in explant cultures Because transcript levels of DNMT1 and MBD2 were shown to be up-regulated significantly in the secretory phase of the menstrual cycle, hormonal regulation of these genes was hypothesized. Therefore, the effect of cultivation with estrogen (E_2) alone or estrogen in

FIGURE 2

Representative photographs of DNMT1 (**A**), MBD2 (**B**) protein, and 5-methylcytosine (**C**) expression in normal endometrium throughout the menstrual cycle (proliferative phase and secretory phase) and in eutopic and ectopic endometrium of patients with endometriosis.

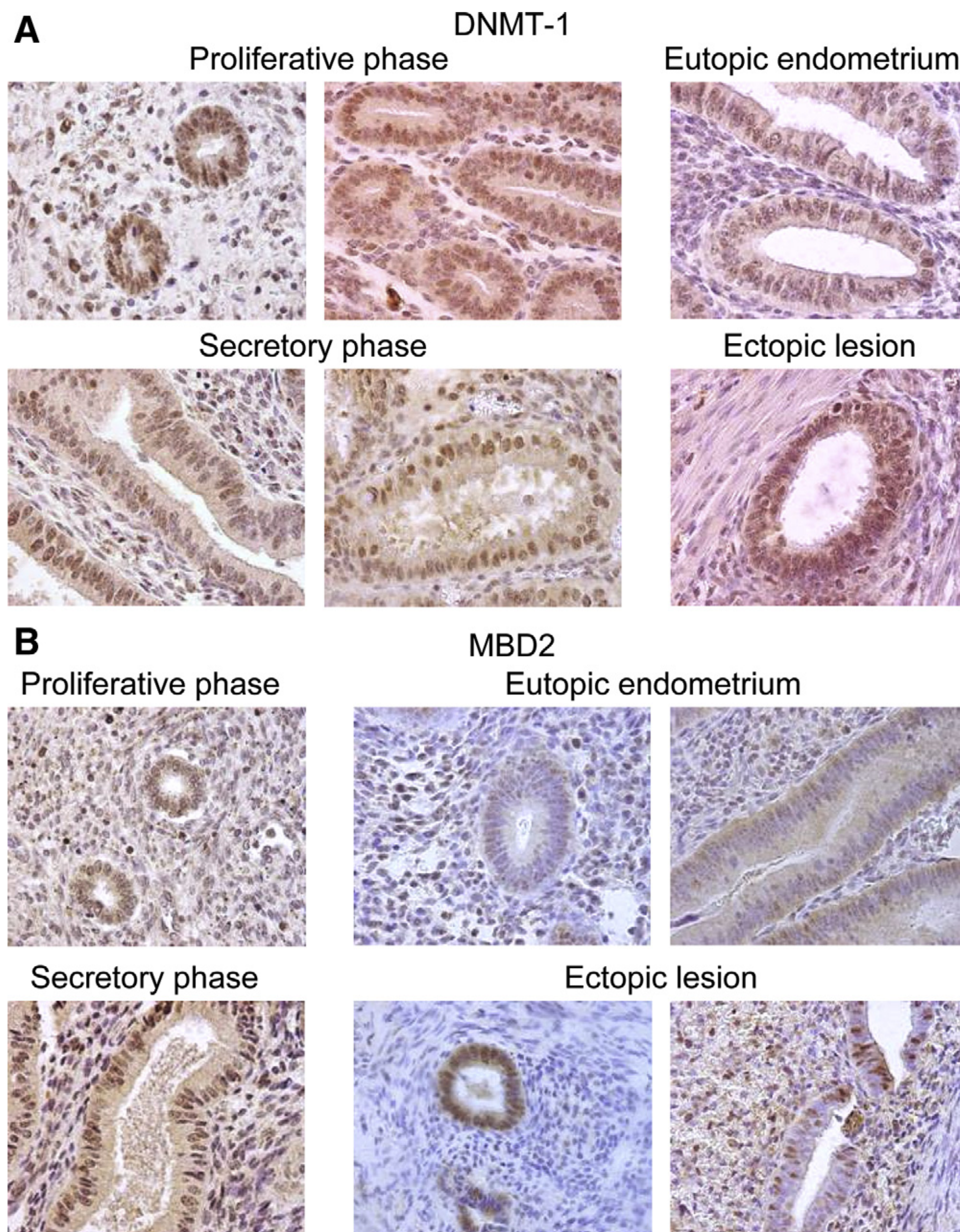

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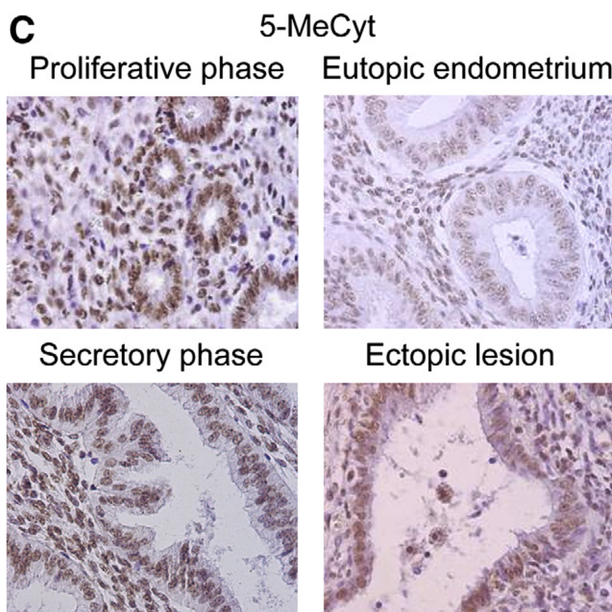
FIGURE 2 Continued



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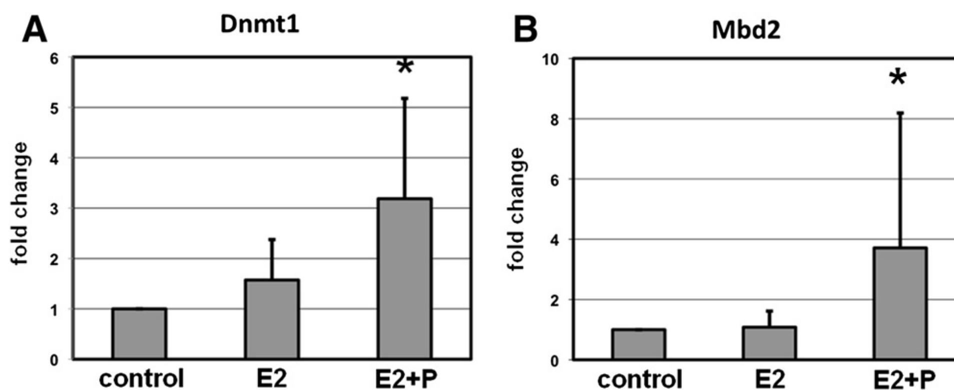
combination with P ($E_2 + P$) was assessed in explant cultures of proliferative endometrium. Expression of DNMT1 was up-regulated significantly by treatment with $E_2 + P$ (P=.021) but not by treatment with E_2 alone (P=.086). The same phenomenon was observed for MBD2: expression levels were shown to be significantly higher when endometrium was cultured with $E_2 + P$ (P=.038) but not after cultivation with E_2 alone (Fig. 3).

Expression levels of deoxyribonucleic acid methyltransferases and methyl-CpG-binding domain proteins in matched samples of eutopic and ectopic endometrium Deoxyribonucleic acid methyltransferase 1 showed significantly lower expression levels in eutopic (P=.007) and ectopic (P=.001) endometrium of patients with endometriosis compared with eutopic endometrium of controls. Expression levels of DNMT3B did not differ between eutopic endometrium of cases and controls but were significantly lower in ectopic endometrium compared with eutopic endometrium of cases (P=.008) and controls (P=.002). The expression of DNMT2 did not vary between the groups. Expression levels of MeCP2 were significantly higher in eutopic endometrium of controls compared with eutopic endometrium of cases (P=.02), but when they were compared with ectopic endometrium no statistically significant difference could be observed. Expression levels of MBD1 on the other hand did not vary between eutopic endometrium of controls and cases but were significantly lower in ectopic endometrium compared with eutopic endometrium of cases (P=.017) and controls (P<.0001). Finally, significantly lower expression levels of MBD2 were observed in eutopic (P=.001) and ectopic (P=.0002) endometrium of cases compared

FIGURE 3

Expression levels of DNMT1 and MBD2 in endometrial explant cultures after cultivation for 24 hours with estrogen (*E2*) or a combination of estrogen and P (*E2+P*). Data are presented as means ± SEM. *P<.05 versus control (uncultured) endometrium.



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with eutopic endometrium of controls (Fig. 1B). Immunoreactivity for DNMT1 (Fig. 2A), MBD2 (Fig. 2B), and 5-methylcytosine (Fig. 2C) was observed in nuclei of epithelial and stromal cells of both eutopic and ectopic endometrium of patients.

DISCUSSION

In the present study, we observed cyclic variations in the expression of DNMT1 and MBD2, with significantly higher expression levels of both enzymes in secretory phase endometrium compared with proliferative and menstrual phase endometrium. Furthermore, we showed that the expression of these genes is regulated by steroid hormones. Strikingly, our findings contradict the report of Yamagata et al. (11), who found significantly reduced levels of DNMT1, DNMT3A, and DNMT3B in the secretory phase of the menstrual cycle. In this study, incubation of cultured proliferative phase endometrial stromal cells with E_2 and P resulted in significant down-regulation of DNMT3A and DNMT3B. The expression of DNMT1 was not influenced under these conditions (11). A possible explanation for this discrepancy could be that the results of these authors were obtained in cultures of isolated endometrial stromal cells, whereas in the present study endometrial explant tissue cultures were used. It has been well documented that stromal-epithelial interactions are indispensable for the hormonal responses of the endometrium (17).

DNMT1 is considered the maintenance DNMT, because its primary function is to copy an already established DNA methylation pattern after replication. On the basis of this functional characteristic, it would be expected that DNMT1 expression levels are highest in the proliferative phase of the menstrual cycle. However, in the present study expression levels of DNMT1 in the secretory phase were shown to exceed those in the proliferative phase. Interestingly, it has been shown that DNMT1-depleted isolated embryonic stem cells remain viable and proliferative but die on induction of differentiation (18–20). Also, targeted deletion of DNMT1 in mouse embryos proved to be lethal, with embryonic death occurring during or after gastrulation when the pluripotent embryonic stem cells begin to differentiate. These findings indicate a role for DNMT1 not only in proliferating cells but also in the subsequent differentiation of cells.

The second protein that was shown to be up-regulated significantly in the secretory phase is MBD2. There is no evidence for an essential embryonic function for any mammalian MBD (21), illustrated by the fact that MBD2- and MeCP2-null mice are viable and fertile and display no overt phenotype (22, 23). However, there is evidence for overlapping functions in differentiation and specification of neural stem cells in culture and in postnatal animals (21). Furthermore, it was shown that MeCP2 and MBD2 expression increases during (terminal) differentiation (24), which is in line with our finding of elevated expression levels of MBD2 in secretory phase endometrium.

In the present study we also show that expression levels of DNMT1 and MBD2 are significantly lower in eutopic and ectopic endometrium of patients with endometriosis compared with eutopic endometrium of controls, whereas MeCP2 expression is lower in eutopic than in ectopic endometrium of patients with endometriosis. Finally, expression levels of DNMT3B and MBD1 are lower in ectopic endometrium compared with eutopic endometrium of both patients with endometriosis and controls.

Our findings do not confirm the observations of Wu et al. (12), who found increased expression levels of DNMT1, DNMT3A, and DNMT3B in ectopic endometrium as compared with disease-free control subjects and eutopic endometrium of women with endometriosis. Possible explanations for this discrepancy may be related to the heterogeneous patient population used by Wu et al. (12) (menstrual, proliferative, and secretory phase endometrium vs. only proliferative phase endometrium in the present study) and the use of isolated endometrial epithelial cells rather than tissue cultures.

As stated earlier, DNMT1, MBD2, and MeCP2 appear to be indispensable for tissue-specific gene expression and differentiation. The findings obtained in the present study may signify that both eutopic and ectopic endometrium of patients with endometriosis has a reduced capacity for differentiation and, hence, decidualization and implantation. Circumstantial evidence in support of this notion has been provided by numerous studies: aberrant expression of progesterone receptor in endometriotic tissue (25–27), the absence of 17βHSD2 in eutopic and ectopic endometrium of patients with endometriosis in the secretory phase (28), and altered expression of HOXA10 in eutopic and ectopic endometrium of patients with endometriosis (29, 30). Furthermore, levels of the decidualization markers prolactin and insulin-like growth factor binding protein-1 are reduced in supernatants from endometrial stromal cells derived from ectopic lesions and from eutopic endometrium of women with endometriosis (31).

In conclusion, we have shown that expression levels of DNMT1 and MBD2 vary throughout the menstrual cycle. Their anticipated dependence on steroid hormones was confirmed in explant cultures prepared from proliferative phase endometrium tissue. The significant reduction in the expression of various DNMTs and MBDs in ectopic endometrium of patients with endometriosis may explain why altered gene promoter methylation has been observed in endometriotic lesions. That these changes in methylation status are also associated with changes in phenotype, that is, gene expression, supports the notion that endometriosis may be an epigenetic disease.

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