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Experimental Murine Endometriosis Induces DNA Methylation and Altered Gene Expression in Eutopic Endometrium[1]

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ABSTRACT

The eutopic endometrium in women with endometriosis demonstrates diminished endometrial receptivity and altered gene expression. It is unknown if the endometrium being defective gives rise to a predisposition toward endometriosis and infertility or, alternatively, if endometriosis causes the altered endometrial receptivity. Here we created experimental endometriosis in mice and examined the expression of several markers of endometrial receptivity in the eutopic endometrium. Methylation of *Hoxa10* was also evaluated as a potential mechanism responsible for altered gene expression. Expression of each gene was measured using quantitative real-time RT-PCR at 14 wk after induction of endometriosis. Expression of *Hoxa10* and *Hoxa11*, which are necessary for endometrial receptivity, were decreased in the endometriosis group. Insulin-like growth factor binding protein-1 (*Igfbp1*) mRNA was decreased in the endometriosis group. However, there was no change in Integrin beta$_3$ (*Itgb3*) mRNA expression. Total progesterone receptor (*Pgr-AB*) was increased in the endometriosis group and the ratio of *Pgr-B* to *Pgr-AB* was increased, indicating a shift from *Pgr-A* to *Pgr-B* expression. Basic transcription element-binding protein-1 (*Bteb1*), official symbol and name *Klf9*, Kruppel-like factor 9, which functionally interacts with *Pgr* in endometrium, was also decreased in the endometriosis group. In addition, hypermethylation of *Hoxa10* in the endometriosis group was shown by methylation-specific PCR and confirmed by bisulfite sequencing. These findings demonstrate that normal endometrium, when placed in an ectopic location to create experimental endometriosis, led to characteristic changes in gene expression in eutopic endometrium. These data suggest the existence of a signal conduction pathway from endometriosis that alters endometrial gene expression through altered *Pgr* signaling and epigenetic programming.

endometriosis, female reproductive tract, gene regulation, Hoxa10*,* Hoxa11*, implantation, methylation,* Pgr*, uterus*

INTRODUCTION

Endometriosis is found in 20% to 50% of women with infertility, which is a principal manifestation of endometriosis [1–3]. Several theories, such as Sampson's theory [4], have been proposed to explain the etiology of endometriosis, but the pathophysiology of endometriosis and the related infertility remains unclear [5]. There are ethical limitations that limit investigation of endometriosis in humans. It is also difficult to monitor the progress of endometriosis in humans continuously, making investigation difficult in humans. Primates such as rhesus monkeys and baboons may be considered ideal animal models to investigate endometriosis because they menstruate in a cyclic pattern and develop endometriosis spontaneously [6–8]. However, rodents have the advantage of low costs and ease of availability and handling. Rodent models of endometriosis have been developed by utilizing i.p. or subcutaneous transplantation of human endometrium in immunocompromised mice and the surgical autotransplantation of endometrium in immunocompetent animals [9–14].

Defective implantation has been demonstrated as one mechanism responsible for endometriosis-associated infertility [15–18]. Previous studies have reported the aberrant expressions of genetic markers of endometrial receptivity in endometriosis [19–22]. Homeobox (*Hox*/*HOX*) genes encode transcription factors that mediate embryonic development [23]. *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* are expressed in the uterus during embryonic development and in the adult [23]. In the human, *HOXA10* and *HOXA11* are expressed in endometrial glands and stroma throughout the menstrual cycle and show a dramatic increase during the mid-luteal phase at the time of implantation in response to estrogen and progesterone [24, 25]. *Hox*/*HOX* genes may affect endometrial development in a way analogous to their role in embryonic development, leading to endometrial growth, differentiation, and receptivity [19, 26]. Mice with a targeted disruption of either the *Hoxa10* or *Hoxa11* gene are sterile due to the loss of endometrial receptivity, yet still produce viable embryos [27–29]. Embryos from these mice implant normally to the uteri of wild-type mice, but embryos from wild-type mice do not implant in *Hoxa10* or *Hoxa11* knockout mice [27–29]. Altered endometrial expression of *Hoxa10* using either *Hoxa10* antisense or a constitutive *Hoxa10* expression construct results in either a decrease or increase in litter size, respectively [30]. These experiments demonstrate cyclic expression of *Hoxa10*/*HOXA10* and *Hoxa11*/*HOXA11* in adult endometrium is necessary for endometrial receptivity. Women with endometriosis fail to upregulate expression of *HOXA10* and *HOXA11* during the window of implantation [19, 31]. In baboons with induced endometriosis, *HOXA10* expression in the eutopic endometrium is decreased [32]. These experiments demonstrate endometriosis leads to altered *HOXA* gene expression and defective endometrial receptivity. Alterations in *Hox*/*HOX* genes may be expected to induce additional alterations in the expression of the downstream target genes of these transcription factors involved in the development of endometrial receptivity.

Implantation in early pregnancy is regulated by the steroid hormones estrogen and progesterone. Progesterone receptor (*Pgr*/*PGR*), a member of the nuclear receptor superfamily, is

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TABLE 1. Primers used in quantitative real-time PCR.

Gene	Primer sequence[a]	Amplicon (bp)	Accession no.	Date of accession	Intron
Hoxa10	F: 5′-gccccttcagaaaacagtaaag-3′ R: 5′-aggtggacgctacggctgatctcta-3′	212	NM_008263	12-APR-2008	Span
Hoxa11	F: 5′-tccagcctcccttctttttg-3′ R: 5′-gtagcagtgggccagattgc-3′	168	NM_010450	11-FEB-2008	Not span
Igfbp1	F: 5′-ccatcagcacctatagcagcat-3′ R: 5′-gcagggctccttccatttct-3′	68	NM_008341	11-FEB-2008	Not span
Itgb3	F: 5′-ggaagcagcgcccagatcac-3′ R: 5′-ttgtccacgaaggccccaaa-3′	254	NM_016780	13-APR-2008	Span
Pgr-AB	F: 5′-ctgtgccttaccatgtggca-3′ R: 5′-ttcaccatgcccgccaggat-3′	389	NM_008829	13-APR-2008	Span
Pgr-B	F: 5′-ggtccccccttgcttgca-3′ R: 5′-caggaccgaggaaaaagcag-3′	121	NM_008829	13-APR-2008	Not span
Klf9	F: 5′-acagtggctgtgggaaagtc-3′ R: 5′-actgcttttccccagtgtg-3′	168	Y14296	18-APR-2005	Span
Actb	F: 5′-gacctctatgccaacacagt-3′ R: 5′-ttgctgatccacatctgct-3′	203	NM_007393	13-APR-2008	Span

[a] F, Forward; R, reverse.

necessary for embryo implantation and subsequent decidualization [33]. Progesterone regulates *HOXA10* and *HOXA11* expression in endometrial glands and stroma [24, 25] through its cognate receptor, PGR [26, 34]. *Hoxa10* is necessary for uterine stromal cell responsiveness to progesterone during implantation and decidualization [35]. Two distinct isoforms of the human progesterone receptor (PGR), termed PGR-A and PGR-B, are encoded by a single gene and they differ in that PGR-B contains N-terminal 164 amino acids [36, 37]. PGR-B functions as a dominant transcriptional activator of progesterone-responsive promoters, whereas PGR-A acts as a dominant repressor of PGR-B and other steroid receptors (estrogen, androgen, and glucocorticoid and mineralocorticoid receptors) [36, 37]. Therefore, the relative expression of *Pgr-A*/*PGR-A* to *Pgr-B*/*PGR-B* determines, in part, the characteristic cellular response to progesterone [36–39].

Klf9, a member of the Sp/Kruppel-like family of transcription factors, functionally interacts with *Pgr-A* and *Pgr-B* to mediate progesterone-responsive gene expression in endometrial epithelial cells [40, 41]. In the uterus of early pregnant *Klf9* knockout mice, numbers of implantation sites are decreased, expression of *Hoxa10* is decreased, and progesterone responsiveness of several uterine genes is decreased [42].

In this study, we developed an experimental mouse model of endometriosis and evaluated expression of *Pgr*, *Hoxa10*, *Hoxa11*, and several downstream target genes related to endometrial receptivity in the eutopic endometrium of mice with experimental endometriosis. We also evaluated the methylation status in the *Hoxa10* gene as a possible mechanism for altered *Hoxa10* expression.

MATERIALS AND METHODS

Development of a Mouse Model of Endometriosis

Eight-week-old CD1 female mice were obtained from Charles River Laboratories (Wilmington, MA) and kept under controlled conditions (12L:12D and 22°C). Laparotomy was performed by midline incision under i.p. anesthesia with xylazine (Lloyd Laboratories, Quezon, Philippines) and ketamin (Fort Dodge Animal Health, Overland Park, KS). In four donor mice, the whole uterus was removed and divided into two horns after washing in PBS. Each uterine horn was transplanted into the abdominal cavity of one recipient mouse. The lumen of each horn was opened longitudinally. Then, the opened horn was transversely divided into two pieces. One piece from each uterine horn was sutured to the parietal peritoneum of the anterior abdominal wall (Vicryl 4–0, Ethicon), and another piece was placed in the abdominal cavity. Finally, the abdominal wall was sutured closed. Experimental endometriosis was created in eight mice. At the same time, seven control mice were created by performing an identical incision, placing the same amount of suture material in a similar location as performed in the experimental group, and closing the abdominal cavity with the same suture material. After 14 wk the uterus was removed from each recipient mouse and divided into two horns. One horn was snap-frozen in TRIzol Reagent (Invitrogen Technologies, Carlsbad, CA) and another horn was frozen immediately at −80°C for later use. This study was approved by the Institutional Animal Care and Use Committee, Yale University, confirming to the U.S. Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training.

Quantitative Real-Time RT-PCR

Total RNA was extracted using TRIzol Reagent with Phase Lock Gel-Heavy (Eppendorf North America, Westbury, NY) according to the manufacturer's instructions and treated using recombinant shrimp DNase (USB, Cleveland, OH) to eliminate DNA contamination. Total RNA (50 ng) was reverse-transcribed using iScript cDNA synthesis kit (Biorad Laboratories, Hercules, CA) for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Real-time PCR was initially performed at 95°C for 3 min and followed by 45 cycles of 95°C for 15 sec, 60.5°C for 20 sec, and 72°C for 25 sec using SYBR Green (BioRad Laboratories) and the MyiQ Single Color Real-Time PCR Detection System (BioRad Laboratories). The specificity of amplified products was confirmed by a melting curve analysis. The oligonucleotide primers are listed in Table 1. Annealing temperature for all primers was 60.5°C. Sterile water was used as a negative control. Messenger RNA expression of *Hoxa10*, *Hoxa11*, Insulin-like growth factor binding protein 1 (*Igfbp1*), Integrin β_3 (*Itgb3*), total *Pgr* (*Pgr-AB*), *Pgr-B*, and *Klf9* was quantified and normalized to β-actin (ACTB) as a control. Then, fold value in expression of each gene was calculated using the $2^{-\Delta\Delta C}{}_{T}$ Method [43]. Each assay was conducted in duplicate and repeated a minimum of three times.

Immunohistochemistry

Tissue was embedded in paraffin, cut into 5-μm sections, and mounted onto slides. Immunohistochemistry for PGR was performed on all slides simultaneously. Slides were first deparaffinized and hydrated through a progression of 10-min xylene and ethanol washes. The tissue was then permeabilized in cold 95% ethanol for 10 min. After being rinsed for 5 min in distilled water, the slides were steamed in 0.01 M sodium citrate buffer for 20 min in order to promote antigen presentation. The slides were then allowed to cool for 20 min in the staining jar containing citrate buffer, followed by a 5-min wash in phosphate buffered saline Tween (PBST). Endogenous peroxidase activity was quenched using a 3% hydrogen peroxide solution for 3 min. After another wash in PBST, the slides were incubated for 1 h at room temperature in a solution of 1.5% normal goat serum in PBST in order to block nonspecific antibody binding. The slides were then incubated at 4°C overnight in a 1:500 dilution of primary antibody in PBST containing 1.5% normal goat serum. The primary antibody used was PR H-190 (sc-7208), purchased from Santa Cruz Biotechnology (Santa Cruz, CA). After three 3-min rinses in PBST, slides were incubated in a biotinylated secondary antibody solution for 1 h at room temperature. The secondary antibody used was goat α-rabbit (BA-1000), purchased from Vector Laboratories (Burlingame, CA) and prepared as a 1:435 dilution in PBST containing 1.5% normal goat serum. Slides were then washed three times in PBST and incubated for 15 min in ABC Elite (Vector). After another three washes in PBST, the slides were incubated for 7 min in

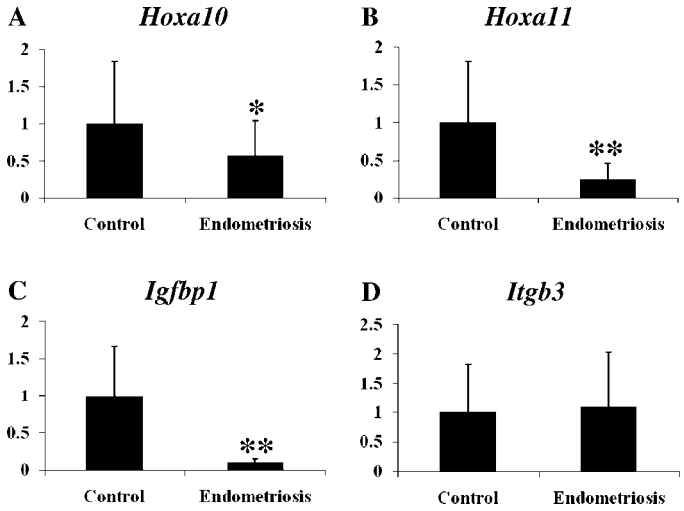

FIG. 1. **A–D**) Expression of *Hoxa10* and its downstream target genes and *Hoxa11* in eutopic endometrium of mice with induced endometriosis. The *y*-axis represents fold change in expression as determined by quantitative real-time PCR and is expressed as mean ± SEM. Asterisk represents statistically significant difference between groups (*$P < 0.05$, **$P < 0.01$).

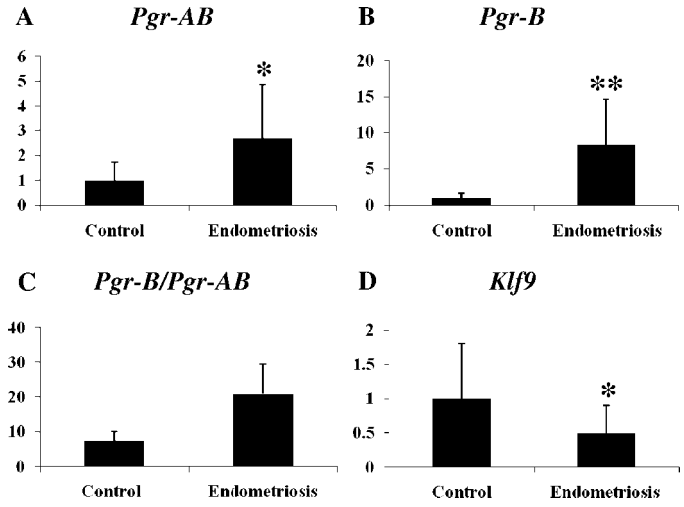

FIG. 2. **A–D**) Expression of *Pgr* and *Klf9* in the eutopic endometrium of mice with induced endometriosis. *Pgr-AB*, *Pgr-B*, ratio of *Pgr-B* to *Pgr-AB*, and *Klf9* were expressed as mean ± SEM. The *y*-axis represents fold change in expression as determined by quantitative real-time PCR. Asterisk represents statistically significant difference between groups (*$P < 0.05$, **$P < 0.01$).

diaminobenzidine, followed by a rinse in distilled water. To counterstain the specific binding to PR, slides were dipped into hematoxylin for 15 sec and then rinsed with distilled water. The slides were then dehydrated through a series of 3-min ethanol and xylene washes. Coverslips were mounted using Permount.

Sodium Bisulfite DNA Modification and Methylation-Specific PCR

Frozen uterine samples were thawed. Genomic mouse DNA was isolated from each uterine sample using the DNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Sodium bisulfite modification using the CpGenome DNA Modification Kit (Chemicon, Temecula, CA) was performed using 1 μg of genomic DNA according to the manufacturer's protocol. In this process, unmethylated cytosine residues are converted to thymine, whereas methylated cytosines remain unchanged [44].

The primers used for the methylation-specific PCR (MSP) amplification were designed from published DNA sequences in the 5′ promoter region of *Hoxa10* [45] using the MethPrimer software [46]: Methylated sequence: forward, 5′-GGGCGAAAGGGGGCGGGT-3′, and reverse, 5′-GCGCCCGCCCGCCGCCT-3′; Unmethylated sequence: forward, 5′-GGGTGAAAGGGGGCTGGT-3′, and reverse, 5′-ACACCCACCCAC-CACCT-3′. The 140-bp region amplified with these primers includes eight CpG sites. PCR amplification of 100 ng bisulfite-treated DNA template was performed in a 50-μl reaction containing 1.5 μl forward and reverse primers and 25 μl SYBR Green. Amplification conditions were: 95°C for 15 min, 40 cycles at 95°C for 30 sec, 62°C (methylated) or 55°C (unmethylated) for 30 sec, and 72°C for 30 sec, followed by a final extension at 72°C for 10 min. CpG Methylated NIH 3T3 Mouse Genomic DNA (New England BioLabs, Beverly, MA) and sterile water were used as positive control and negative control, respectively. The PCR products were separated by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

Bisulphite Sequencing of Hoxa10 *Gene*

The primers used for the bisulfite genomic sequencing PCR amplification were designed from mouse DNA sequences that were conserved in the 5′ promoter region of human *HOXA10* gene [47] using the MethPrimer software [46]: forward, 5′-TATTTTGAGGTAGTTTTTATAGTTT-3′, and reverse, 5′-ATAACCCTTTCTAACTAACATT-3′. The 271-bp region amplified with these primers includes 20 CpG sites. PCR amplification of 100 ng bisulfite-treated DNA template was performed in a 50-μl reaction containing 1.5 μl forward and reverse primers, 3 μl of 25 mM Mg^{2+}, 5 μl of 10× buffer, 1 μl of 1.25 mmol/L deoxynucleotide triphosphates, and 0.5 μl of HotStarTaq DNA polymerase (Qiagen). Amplification conditions were: starting at 95°C for 15 min, 40 cycles at 95°C for 30 sec, 53°C for 30 sec, and 72°C for 30 sec followed by a final extension at 72°C for 10 min. Direct sequence analyses of PCR products were carried out on Applied Biosystems 3730 capillary instruments (Applied Biosystems, Foster, CA).

Statistical Analyses

Statistical analyses were performed using SigmaStat (Systat Software, Point Richmand, CA). Statistical differences in expression of genes between the endometriosis model group and control group were calculated by a Mann-Whitney Rank Sum Test. Using bisulphite sequencing, *Hoxa10* promoter sequences were analyzed for methylation status in the endometriosis group ($n = 7$) and control group ($n = 4$). Since there are 20 CpG sites in the 5′ promoter region of Hoxa10, we analyzed $20 \times 7 = 140$ CpG sites, and $20 \times 4 = 80$ CpG sites, respectively, in each experimental group. The percentage of methylated CpGs was calculated by the number of methylated CpGs divided by the total number of CpGs analyzed. The difference in methylation frequencies of Hoxa10 gene between different experimental groups was calculated by a chi-square test. $P < 0.05$ was considered statistically significant.

RESULTS

Altered Gene Expressions in Mice after Experimental Induction of Endometriosis

Gene expression in the eutopic endometrium of mice with experimentally induced endometriosis and control mice at 14 wk after the induction of endometriosis were measured by quantitative real-time PCR. *Hoxa10* and *Hoxa11* mRNA expression decreased 0.57-fold ($P = 0.046$) and 0.25-fold ($P < 0.001$) in the endometriosis group compared to the control group, respectively (Fig. 1, A and B). Expression of *Igfbp1* and *Itgb3*, downstream target genes of *Hoxa10*, were measured. *Igfbp1* mRNA, which is positively regulated by *Hoxa10* [48, 49], was decreased 0.09-fold ($P < 0.001$) in the endometriosis group ($n = 7$) compared to the control group (Fig. 1C). There was no significant change (1.1-fold, $P > 0.05$; Fig. 1D) in *Itgb3* mRNA expression. Total *Pgr* (*Pgr-AB*) and *Pgr-B* mRNA increased 2.7-fold ($P = 0.016$) and 8.39-fold ($P = 0.006$) in the endometriosis group compared to the control group, respectively (Fig. 2, A and B). The ratio of *Pgr-B* to *Pgr-AB* was increased (7.53 vs. 20.98) in endometriosis. This increase was out of proportion to the increase in *Pgr-B*, suggesting that *Pgr-A* expression was decreased (Fig. 2C). *Klf9*, which functionally interacts with *Pgr* in endometrium

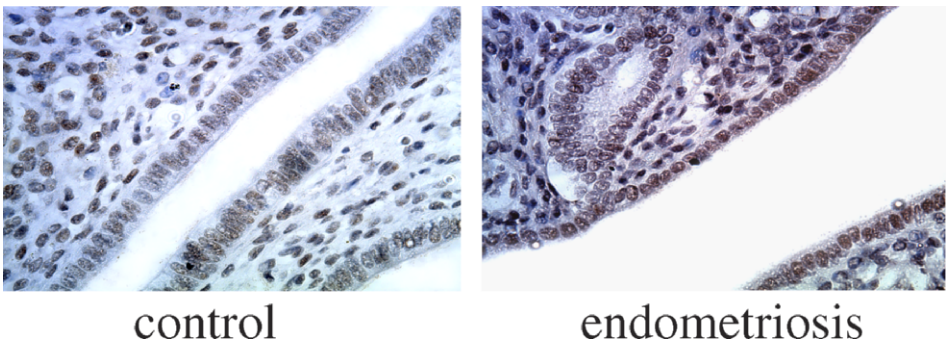

FIG. 3. Expression of PGR protein in the eutopic endometrium of mice with induced endometriosis. Immunohistochemistry was used to demonstrate higher levels of PGR expression in the endometrium of mice with induced endometriosis compared to controls. Original magnification ×600.

[40–42], was decreased 0.50-fold ($P = 0.043$) in the endometriosis group compared to the control group (Fig. 2D). The increase in PGR protein expression was confirmed using immunohistochemistry as demonstrated in Figure 3.

Methylation of the Hoxa10 *in an Experimental Model of Endometriosis*

To determine whether hypermethylation of *Hoxa10* induced a decrease in *Hoxa10* expression in endometriosis, methylation in the 5′ promoter region of *Hoxa10* was measured. Several CG-rich regions of the 5′ promoter region of *Hoxa10* were measured using both MSP and bisulfite sequencing (accession no. and date of accession: AF246720, 19 July 2002 for MSP; NM_008263, 12 April 2008 for bisulfite sequencing; Fig. 4, A and B). Using MSP, one CpG site showed only methylated bands without any unmethylated bands, suggesting completely methylated CpG in endometriosis. At this same site only unmethylated bands were detected in controls (Fig. 5). A negative control that used non-bisulfite-treated DNA failed to show a band using methylation-specific primers (Fig. 5).

The methylation status of the *Hoxa10* gene was further evaluated in the 5′ promoter region by bisulfite sequencing of the PCR product. Twenty CpG sites were analyzed from each sample. CpG was considered as partially methylated when the proportion of C nucleotide signal was less than 90% of the sum of C and T nucleotides at a given position. Partial methylation of 15 CpGs was shown in the endometriosis group ($n = 7$, a total of 140 CpG sites), and one of the partially methylated CpGs was highly methylated even though we did not consider it as methylated for the purpose of this analysis. In contrast, all of the CpGs were unmethylated in the control group ($n = 4$, a total of 80 CpG sites). Figure 6, A and B, shows the chromatograms of the 5′ promoter region of *Hoxa10* in each group. Percentages of methylated CpGs were calculated in both groups. The endometriosis group demonstrated methylation at a high percentage of CpG sites compared to the control group (0% vs. 10.7%, control vs. endometriosis, $P = 0.006$; Fig. 6C).

A

GGGCGAAAGGGGGCGGGCAGGGAAGCTCGGTTTAGGAAAAGACTTCCTTTGGGGT
GGTTAAAGATTAACCAAAGTCTCTCAAGTTGCCAGAGTGAGTCTAGCCAGGAGAACT
GCTTGCAGGCAGGCGGCGGGCGGGCGCG

B

TATTTTGAGGTAGTTTTTATAGTTT CGGTTTTTGAGTTATAGGTGTTAGGCGTGCGTT
TTTTGGTTTATTAATATAGATTATATATTTATATTAATCGCGGGTTTCGAGGGCGTTTT
TAGAGAGCGGTTTCGCGTTTACGAAATTAAATTGGGAGTGGTCGCGTGGAAATTTTG
GTTCGGGATTGGTTGTAAGCGTTCGTCGCGGTGCGGGGGGGATTATTAATCGTATTT
AGTATGTTTTGTATAAGAAATGTTAGTTAGAAAGGGTTAT

FIG. 4. The mouse nucleotide sequences subjected to MSP (**A**) and bisulphate sequencing (**B**) in the 5′ promoter region of *Hoxa10* gene.

DISCUSSION

Experimentally induced endometriosis lowers fecundity in rats, similar to the effect of endometriosis in humans [50]. *Hoxa10* expression, a mediator of endometrial receptivity, is decreased in eutopic endometrium in humans with endometriosis, as well as in baboons with induced endometriosis [32]. Here we demonstrate that *Hoxa10* and *Hoxa11* expression was down-regulated in the eutopic endometrium of mice with induced endometriosis. Altered expression of *Hoxa*/*HOXA* genes in animal models of endometriosis is consistent with aberrant expression of the *HOXA10* and *HOXA11* genes in women with endometriosis [19, 31, 47].

We also demonstrated altered expression of downstream target genes of the *Hoxa10* gene in the eutopic endometrium of mice with induced endometriosis. *HOXA10* can up-regulate *IGFBP1* promoter activity with or without Forkhead transcription factor-1 (*FOXO1*) in endometrial cells [48, 49], and *IGFBP1* secreted by decidualizing endometrial stromal cells is reduced in women with endometriosis [51]. Our data demonstrate that *Igfbp1*, a marker of decidualization, is down-regulated in the mouse model of endometriosis, consistent with previous reports. However, recently, it has been reported that *HOXA10* decreases *IGFBP1* expression in decidualizing cells from baboons with endometriosis [32]. This result may reflect the cell culture model as opposed to the in vivo results obtained here and in humans.

ITGB3, which is a marker of endometrial receptivity, is decreased in the endometrium of women with endometriosis [52]. We have previously identified that *ITGB3* is directly up-regulated by *HOXA10* through a 41-bp 5′-regulatory element in the human endometrium [53]. However, our data demonstrated no change in *Itgb3* expression in the eutopic endometrium of mice with induced endometriosis. This result is in contrast to results obtained in baboons with endometriosis, which showed decreased *ITGB3* expression [32]. Interestingly, our mouse model and baboon model with induced endometriosis are consistent with regard to *Hoxa10* mRNA expression; however, mRNA expression of its downstream target genes differs between models. It is most likely that differences in gene expression are explained based on the duration of endometri-

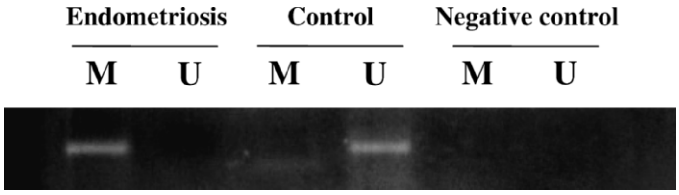

FIG. 5. Methylation status in the 5′ promoter region of *Hoxa10* gene by MSP from the endometriosis group and control group. M, Methylated primer; U, unmethylated primer.

osis. We obtained endometrial tissues at 14 wk after the induction of endometriosis in the mouse model, much earlier than in the baboon model (16 mo) and in humans. Here we likely examined an earlier stage of endometriosis compared to reports in baboons and humans.

The relative expression of *Pgr-A*/*PGR-A* to *Pgr-B*/*PGR-B* modifies progesterone responsiveness in target cells [36–39]. Here we demonstrated an altered ratio of *Pgr-B* to *Pgr-A* mRNA and increased PGR protein expression in the eutopic endometrium of mice with induced endometriosis compared to controls. These findings suggest that whereas total *Pgr* and *Pgr-B* were increased, *Pgr-A* was expressed at a lower level in animals with endometriosis. *PGR* expression is also similarly altered in the eutopic endometrium of baboons with induced endometriosis [54]. A recent microarray-based study in women with moderate to severe endometriosis also reported similarly increased total PR in women with endometriosis compared to controls [55]. We therefore believe that our model most closely reflects moderate to severe disease in women. He we show that despite increased total *Pgr, Pgr-A* is significantly decreased.

In normal human endometrial epithelium, both *PGR-A* and *PGR-B* are increased by estrogen during the proliferative phase but are reduced during the secretory phase under the influence of rising serum progesterone levels [56, 57]. *PGR-A* predominates throughout the cycle in the stroma, suggesting a function for this isoform in progesterone-mediated stromal decidualization [2, 57]. *Pgr-A* knockout mice have defective implantation based on loss of progesterone-regulated expression of several genes associated with uterine receptivity [38]. Treatment of ovariectomized *Pgr-A* knockout mice with estrogen and progesterone induces progesterone-dependent proliferative activity mediated through *Pgr-B* in uterine epithelium, suggesting that *Pgr-A* is essential in order to diminish both progesterone (acting via *Pgr-B*) and estrogen-mediated proliferative responses in uterus [38]. *Pgr-B* knockout female mice are fertile and sustain a normal pregnancy, suggesting normal uterine responses to progesterone [39]. Therefore, those studies demonstrate that only *Pgr-A,* but not *Pgr-B,* is necessary to elicit progesterone-dependent reproductive responses [38, 39]. Progesterone, which inhibits estrogen-mediated mitosis in endometrium [58], mediates its antiproliferative activity through *Pgr-A* [38, 59]. The antiproliferative effects of progesterone are less pronounced in endometriosis compared with normal endometrium [58]. Therefore, we suggest that the relatively low expression of *Pgr-A* in the eutopic endometrium of mice with induced endometriosis may result in a defect in proliferation. These data may also explain the decrease in expression of *Hoxa10* and *Hoxa11*, which are progesterone-responsive genes. Taken together, these results suggest increased total PGR expression is accompanied by a decreased expression of the critical PGR-A in endometriosis. The altered PGR expression leads to diminished PGR response and decreased expression of progesterone-responsive genes.

Recently, it has been reported that *Klf9* expression in normal murine endometrium during early pregnancy is predominantly localized to stromal cells and temporally coincides with *Pgr-A*-dependent decidual formation at the time of implantation, suggesting a functional contribution of *Klf9* to *Pgr-A* action in uterine endometrial stromal cells [42]. Here we demonstrated that *Klf9* is down-regulated in the eutopic endometrium of mice with induced endometriosis. Therefore, we suggest that decreased *Klf9* and *Pgr-A* mRNA expression may contribute to defective endometrial receptivity in mice with induced endometriosis. In our study, *Klf9* is down-regulated in concert with a decrease in *Hoxa10* expression in the eutopic endometrium of mice with induced endometriosis, corresponding to a previous report showing decreased *Hoxa10* expression in *Klf9* knockout mice [42].

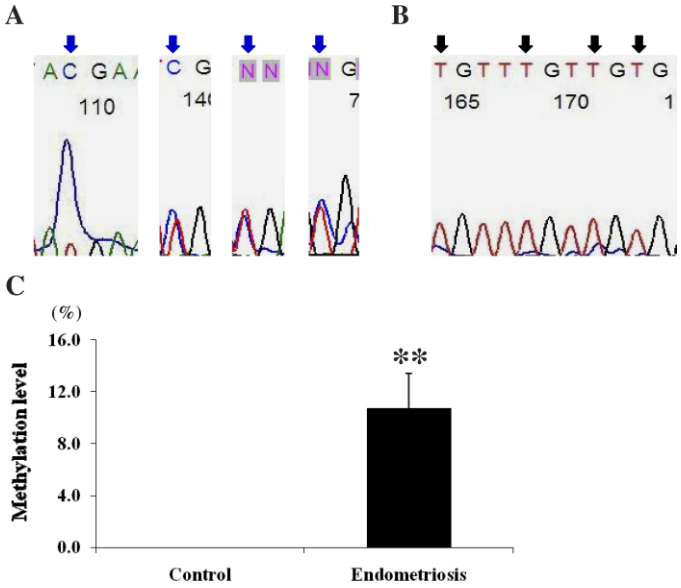

FIG. 6. The chromatograms from bisulfite sequencing of 5′ promoter region of *Hoxa10* in the endometriosis group (**A**) and the control group (**B**). Blue arrows indicate partially methylated CpGs, and black arrows indicate unmethylated CpGs. **C**) Methylation level (mean ± SEM) in 5′ promoter region of *Hoxa10* gene by bisulfite sequencing in endometriosis group and control group. Asterisks represent statistically significant difference between groups (**$P < 0.01$).

DNA methylation is an epigenetic modification of DNA that has an important role in embryonic development, tumorigenesis, aging, and other diseases [60–62]. When promoter CpG islands become methylated, the associated gene typically becomes permanently silenced or repressed due to suppressed transcriptional activity. Recent studies have reported *HOXA10* methylation is one possible mechanism by which *HOXA10* levels are decreased in endometriosis. *HOXA10* in the eutopic endometrium of both women with endometriosis and baboons with induced endometriosis is hypermethylated compared with those without endometriosis [47, 32]. The expression levels of three genes that code for DNA methyltransferase (*DNMT1*, *DNMT3A*, and *DNMT3B*) are overexpressed in the epithelial component of endometriotic implants in humans [63]. Here we demonstrate that 5′ promoter region of *Hoxa10* is hypermethylated in the eutopic endometrium of mouse with induced endometriosis. *Hoxa10*/*HOXA10* methylation may, in part, explain mechanism through which *Hoxa10*/*HOXA10* is down-regulated in endometriosis.

The eutopic endometrium in women with endometriosis demonstrates altered endometrial receptivity and altered gene expression. Similarly, we demonstrate significant changes in multiple markers of endometrial receptivity in the eutopic endometrium after induction of endometriosis, suggesting that the mouse model is useful in investigating endometriosis. These findings also suggest that normal endometrium can develop endometrial defects in endometriosis; an abnormal endometrium is not a prerequisite for the development of endometriosis or associated abnormalities. Finally these data also suggest the existence of altered signal conduction pathways resulting from endometriosis that change endometrial gene expression. These pathways include altered progesterone receptor, cofactor, and target gene expression levels as well as epigenetic transcriptional repression.

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