The Endometrial Response to Chorionic Gonadotropin Is Blunted in a Baboon Model of Endometriosis

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Endometriosis-associated infertility has a multifactorial etiology. We tested the hypothesis that the endometrial response to the early embryonic signal, human chorionic gonadotropin (hCG), alters over time in a nonhuman primate model of endometriosis. Animals with experimental or spontaneous endometriosis were treated with hCG (30 IU/d), from d 6 after ovulation for 5 d, via an oviductal cannula. Microarray analysis of endometrial transcripts from baboons treated with hCG at 3 and 6 months of disease (n = 6) identified 22 and 165 genes, respectively, whose levels differed more than 2-fold compared with disease-free (DF) animals treated with hCG ($P < 0.01$). Quantitative RT-PCR confirmed abnormal responses of known hCG-regulated genes. *APOA1*, *SFRP4*, and *PAPPA*, which are normally down-regulated by hCG were up-regulated by hCG in animals with endometriosis. In contrast, the ability of hCG to induce *SERPINA3* was lost. Immunohistochemistry demonstrated dysregulation of C3 and superoxide dismutase 2 proteins. We demonstrate that this abnormal response to hCG persists for up to 15 months after disease induction and that the nature of the abnormal response changes as the disease progresses. Immunohistochemistry showed that this aberrant gene expression was not a consequence of altered LH/choriogonadotropin receptor distribution in the endometrium of animals with endometriosis. We have shown that endometriosis induces complex changes in the response of eutopic endometrium to hCG, which may prevent the acquisition of the full endometrial molecular repertoire necessary for decidualization and tolerance of the fetal allograft. This may in part explain endometriosis-associated implantation failure. **(*Endocrinology* 151: 4982–4993, 2010)**

Endometriosis, the presence of endometrial glands and stroma outside the uterine cavity, occurs in 10% of women of reproductive age and may present with pelvic pain, dyspareunia, or infertility (1). However in patients presenting with subfertility, the prevalence of endometriosis is 30–40% (1, 2). Multiple factors have been implicated in endometriosis-associated infertility (3, 4). Eutopic endometrium from women with endometriosis exhibits defects that include ultrastructural abnormalities and alterations in molecular markers of endometrial receptivity as well as aberrant decidualization (3, 5–7). Patients with endometriosis also show reduced rates of follicular growth, reduced functional capacity of the preovulatory follicle, reduced fertilization rates, abnormal preimplantation embryonic development, and altered early luteal function (8–10). The hypothesis that endometriosis reduces implantation rates

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Abbreviations: ASMA, α-Smooth muscle actin; C3, complement factor C3; DF, disease free; FKBP52, FK506-binding protein 4; hCG, human chorionic gonadotropin; IVF, *in vitro* fertilization; LHCGR, LH/choriogonadotropin receptor; PO, postovulation; qRT-PCR, quantitative RT-PCR; SOD2, superoxide dismutase 2.

has also been addressed. Templeton *et al.* (11), reviewing outcome data from the Human Fertilization and Embryology Authority database, concluded that the presence of endometriosis was not associated with a reduction in *in vitro* fertilization (IVF) pregnancy rates. However, in these data from the early 1990s, the overall live-birth rate per cycle of treatment was only 13.9 and 8% of the patients who had two or more causes of infertility. Therefore, the impact of endometriosis on implantation may have been underestimated. A subsequent metaanalysis of 22 studies, which investigated outcome of IVF treatment for patients with endometriosis, concluded that the success rates for women with endometriosis were significantly lower compared with women without the disease (12). Specifically implantation rates were lower in patients with endometriosis compared with controls with tubal factor subfertility [12.7 *vs.* 18.1%; adjusted odds ratio 0.86 (0.85–0.88)].

Endometriosis is expensive to treat and difficult to study because there are significant delays in the diagnosis and variations in symptomatology and disease progression for affected women (13, 14). Also, at the time of clinical presentation, most women have established disease, making longitudinal studies impossible. Thus, the baboon (*Papio anubis*) has been developed as a model for the study of this enigmatic disease (15, 16). We have previously shown that after the experimental induction of endometriosis, the eutopic endometrium in these animals shows multiple defects, suggesting that the presence of endometriosis itself induces endometrial abnormalities (17). We have further shown in a baboon model of simulated pregnancy that human chorionic gonadotropin (hCG), a major early, embryo-derived signal, induces alterations in eutopic endometrial gene expression (18). The genes regulated by hCG modulate embryo attachment, extracellular matrix remodeling, and the immune response around the implanting blastocyst.

In preliminary investigations in baboons with experimentally induced endometriosis, we demonstrated that hCG failed to induce progestogen-associated endometrium protein, previously known as glycodelin A, the most abundant secretory product of early pregnancy (19). Therefore, we hypothesized that the endometrial response to hCG is altered in baboons with endometriosis. In this study we investigated how the global endometrial response to *in vivo* hCG treatment in baboons alters over time after the induction of experimental endometriosis. We used Illumina Expression Beadchips (Little Chesterford, UK) to determine the endometrial response to hCG in disease-free animals (DF+hCG). This was compared with the responses induced by hCG in animals 3 and 6 months after the induction of endometriosis, referred to as the 3mo+hCG and the 6mo+hCG groups, respectively (Fig. 1). This allowed us to study how the responses to this early embryonic signal evolved over time, after disease induction.

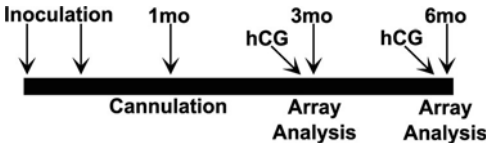

FIG. 1. Experimental paradigm in the nonhuman primate of simulated early pregnancy in an induced model of endometriosis. Baboons (*P. anubis*) were inoculated with menstrual endometrium during two consecutive menses. In the subsequent cycle, a cannula was secured into the oviduct during the window of implantation. At this time eutopic endometrium was harvested by endometrectomy serving as unstimulated control endometrial tissue. Three and six months (mo) after induction of disease, hCG was infused into the oviductal lumen, mimicking the secretory behavior of the preimplantation embryo. Eutopic endometrium was subsequently harvested after 4–5 d of hCG infusion. Microarray analysis was performed to determine whether eutopic endometrium responded aberrantly to the early embryonic signal as a proposed mechanism for endometriosis-associated infertility.

Materials and Methods

Materials

Recombinant hCG was obtained from EMD Serono (Rockland, MA). Mouse monoclonal antibody against α-smooth muscle actin (ASMA) was obtained from Dako (Carpinteria, CA). Mouse monoclonal antibodies against complement factor C3 (C3) and superoxide dismutase 2 (SOD2) were obtained from Gene Tex, Inc. (San Antonio, TX). Antibody against LH/choriogonadotropin receptor (LHCGR) was raised in male rabbits against a synthetic peptide corresponding to amino acids 257-271 within the extracellular domain of the human LHCGR (National Center for Biotechnology Information, Bethesda, MD; accession no. S57793) (20, 21).

Animals

All experimental procedures were approved by the Animal Care Committee of the University of Illinois (Chicago, IL). Eighteen female *P. anubis* baboons with documented regular menstrual cycles were used in this study, as described below.

Induction of endometriosis

Endometriosis was experimentally induced in four baboons, which had not undergone previous surgical intervention, by ip inoculation with menstrual endometrium on two consecutive menstrual cycles, as previously described (15, 17). One month after the induction of disease a polyvinyl cannula (Access Technologies, Skokie, IL) was inserted into the oviduct and exteriorized through a sc flank incision, as described previously (22). Eutopic endometrium was harvested at this 1-month time point by endometrectomy. The progression of disease was monitored in each animal by consecutive laparoscopies and video recording at 3, 6, 12, and 15 months after inoculation, during the window of uterine receptivity [d 9–11 postovulation (PO) in the baboon]. At the time of laparoscopy, the number, color (red, blue, chocolate, white, or mixed pigmentation) and position of each visible

lesion were documented by video recording. The number and type of lesions ranged between animals, but on average we observed four red, three blue, two chocolate, two white, and two lesions of mixed pigmentation during each laparoscopy. After each laparoscopy a laparotomy was performed, and eutopic endometrium was harvested by endometrectomy. Lesions were not harvested from baboons with disease. However, we have previously documented that 67% of ectopic lesions harvested from baboons induced with experimental endometriosis contained both endometrial glands and stroma (17). The presence of peritoneal fluid (clear or bloody), extent of peritoneal adhesions, the level of surface vasculature on the peritoneal wall and organs, and scar tissue was noted. A corpus luteum was seen in all animals.

Spontaneous endometriosis

Two baboons with spontaneous endometriosis were included in this study. The first animal was thought to have endometriosis for approximately 6 months because it had undergone a negative laparoscopy 6 months before and a positive laparoscopy 3 months before this study. The second animal had a positive laparoscopy 6 months before inclusion in this study.

Collection and processing of tissue

Blood samples were collected daily from d 7 through d 16 after menstruation (where d 1 was the first day of menstruation) of menstrual cycles during which surgery was to be performed. Serum 17β-estradiol was measured by RIA (Diagnostic Systems Laboratories, Webster, TX). The serum 17β-estradiol peak was taken as d -1 of ovulation and the day of ovulation was designated as d $+1$ (22). On d 6 PO, a primed Alzet osmotic minipump (2ML1; Alzet, Palo Alto, CA) containing recombinant hCG (300 IU in 2.4 ml saline) was attached to the exteriorized cannula. The flow rate of the pump was 10 μl/h; the hourly infusate was 1.25 IU, as previously described (22). hCG was infused at 3 months endometriosis [n = 4 animals with induced disease (3mo+hCG)], 6 months endometriosis [n = 6, four animals with induced disease and two animals with spontaneous disease (6mo+hCG)], 12 months endometriosis [n = 4 animals with induced disease (12mo+hCG)], and 15 months endometriosis [n = 3 animals with induced disease (15mo+hCG)], as described in Table 1. Endometrial tissue was subsequently harvested by endometrectomy between d 9 and 11 PO, which corresponds to the approximate time of implantation in the baboon. Harvested eutopic endometria, containing both functionalis and basalis layers, were either snap frozen in liquid N_2 for RNA extraction or fixed in 10% buffered formalin for 24 h at room temperature for immunohistochemical and morphological analysis.

TABLE 1. Macroscopic endometrial response to hCG infusion

	3 months	6 months	12 months	15 months
Induced animal 1	−	−	−	−
Induced animal 2	−	−	−	−
Induced animal 3	−	−	−	+
Induced animal 4	−	+	+	NCG
Spontaneous animal 1	−			
Spontaneous animal 2	−			

Animals with spontaneous endometriosis were sampled at one time point only. +, Response to chorionic gonadotropin; −, no response to chorionic gonadotropin; NCG, no hCG infusion was performed.

DF control animals

An additional 12 control, DF baboons were included in this study. Six animals were subjected to hCG infusion between d 6 and d 10 PO, as described above and are referred to as the DF+hCG group. The remaining six animals represent control, unstimulated animals and are referred to as the DF-hCG group. Although control animals did not undergo multiple laparoscopies, they were subjected to multiple endometrectomies. Of the 12 control DF animals, this was the first endometrectomy for one animal, six animals had undergone one previous endometrectomy, two animals had undergone two previous endometrectomies, one animal had undergone three endometrectomies, and two animals four endometrectomies. Thus, the 12 control animals had on average undergone two previous endometrectomies at the time of tissue sampling; a similar number of surgeries in comparison with the animals with endometriosis, whereby in the 6-month treatment, the animals had undergone two previous endometrectomies.

RNA extraction

Each harvested eutopic endometrial sample was homogenized in Trizol (Invitrogen Life Technologies, Carlsbad, CA). Total RNA was purified with chloroform/isoamyl alcohol. RNA quality was assessed by loading 200 ng of total RNA onto a RNA Labchip and analyzed on an A2100 Bioanalyzer (Agilent Technologies, South Queensferry, UK).

Microarray hybridization and data analysis

Total RNA (0.4 μg) from each sample was used to synthesize double-stranded cDNA and subsequently biotin-labeled cRNA, using the Illumina TotalPrep RNA amplification kit. The labeled cRNA was quantified by NanoDrop (Fisher Scientific, Loughborough, UK), and 1.5 μg of the target was hybridized to each array according to the Illumina Sentrix Human-6_V2 protocol. Samples were hybridized for 17 h at 58 C and then washed according to the protocol and scanned with a BeadArray reader (Illumina). Data were extracted from the scanned images using the BeadStudio software (Illumina).

Microarray data analyses were performed using Biometric Research Branch Array Tools developed by Dr. Richard Simon and Amy Peng (National Cancer Institute, Biometric Research Branch, Division of Cancer Treatment and Diagnosis; http://linus.nci.nih.gov/BRB-Array-Tools.html). An intensity filter was used to remove very low signal spots (signal < 50) and median-array normalization was performed. Scatter plots were visually analyzed for the dispersion of the data and unsupervised clustering was performed to detect sample outliers. The Class Comparison between Groups of Arrays tool was used to calculate a random-variance *t* test for each gene using the normalized logged data. The random-variance *t* test permits the sharing of information among genes, including within-class variation, without assuming that all genes have the same variance (23). A stringent significance threshold ($P < 0.01$) was used to limit the number of false-positive findings and a fold change ($0.5 <$ or > 2) was chosen to produce a manageable number of genes.

Quantitative real-time PCR

Verification of the microarray results was performed using quantitative RT-PCR (qRT-PCR) as previously described (18).

Verification was performed for four genes, using either SYBR green or qRT-PCR: apolipoprotein A-I (*APOA1*, NM_000039.1, forward, GGC AGA GAC TAT GTG TCC CAG TT, reverse, GTC CCA GTT GTC AAG GAG CTT T), pregnancy-associated plasma protein (*PAPPA*, NM_002581.3, forward, TGG CCT CCA TCC TAC ATC TC, reverse, ATC GCC ACA GTA CCC ACT TC), and α1-antichymotrypsin (*SERPINA3*) and soluble frizzled-related protein 4 (*SFRP4*, NM_003014, forward, CAC ATC CTG CCC CAT CAA G, reverse, GCA ATT TTC AAG AAG CAT CAT CCT, probe, TTC TCA TCA TGT GTT ACG AGT GGC GCT C). The obtained expression values were normalized against those from control ribosomal 18S (NR_003286.2, forward, CCT GCG GCT TAA TTT GAC TC, reverse, ATG CCA GAG TCT CGT TCG TT) to account for differing amounts of starting material. Expression levels in the control and treated tissues were compared using the nonparametric Mann-Whitney *U* test or the Kruskal Wallis ANOVA. Differences were considered statistically significant when $P < 0.05$.

Immunohistochemistry

Formalin-fixed endometrial tissues were embedded in paraffin; 5-μm sections were examined for the immunolocalization of ASMA, C3, SOD2, and the LHCGR, as previously described (18, 22, 24). Briefly, after antigen retrieval with antigen unmasking solution (Vector Laboratories, Burlingame, CA) and a Decloaking Chamber electric pressure cooker (Biocare Medical, Walnut Creek, CA), endogenous peroxidase activity was blocked with 0.3% H_2O_2. After blocking with 3% normal serum, sections were incubated in primary antiserum at 4 C. Immunostaining was developed with Vector ABC Elite peroxidase Vectastain kits (Vector Laboratories) and 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained in Gill's hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Pittsburgh, PA). Subsequent sections were stained with Gomori's Trichrome (25). Stained sections were examined on an Olympus Provis microscope (Southend-on-Sea, UK) and documented using a Zeiss AxioCam HRC camera (Welwyn Garden City, UK) and Axiovision software.

Results

Morphological characterization of eutopic endometrial response to CG infusion in baboons with experimental endometriosis

We have shown that the infusion of a low dose of hCG to mimic the early preimplantation embryo, in DF animals, results in formation of a luminal epithelial plaque, the induction of ASMA in stromal fibroblasts and up-regulation of progestogen-associated endometrium protein in glandular epithelial cells. These responses are suppressed in baboons with experimentally induced endometriosis (15, 22). In this current study, the endometrium of all animals in the DF+hCG group showed the expected morphological responses to hCG. Conversely, all four animals in the 3mo+hCG group and five of the six animals in the 6mo+hCG group failed to elicit a luminal epithelial plaque response or show expression of ASMA in the subluminal stromal fibroblasts (Table 1 and Fig. 2). The animals with induced endometriosis were also treated with hCG at 12 and 15 months after disease induction (12mo+hCG and 15mo+hCG, respectively). At 12 months of disease, three of four hCG-treated baboons failed to develop an epithelial plaque or ASMA immunostaining in response to hCG. In the 15mo+hCG group, two of three baboons failed to demonstrate a morphological response to hCG (Table 1). Thus, the presence of endometriosis continues to affect the endometrial response to hCG, up to 15 months after induction of the disease.

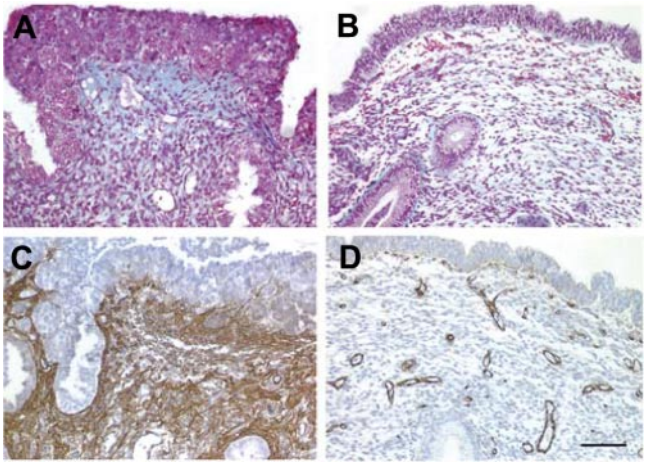

FIG. 2. Morphological characterization and ASMA immunostaining of eutopic endometrium after hCG infusion. hCG infusion in DF baboons (A and C) resulted in the generation of a luminal epithelial plaque (A) and the induction of ASMA in the endometrial stroma (C). In animals with 6 months endometriosis (B and D) hCG failed to elicit both an epithelial plaque (B) or ASMA production (D) in endometrial stromal cells. *Bar*, 100 μm.

The genomic response to hCG is altered in baboons with experimental endometriosis

To examine differences in RNA transcripts induced in response to hCG, endometrial cRNAs from animals in the 3mo+hCG (n = 4) and 6mo+hCG (n = 6) groups were hybridized to Illumina Beadchips. These were compared with cRNAs from the DF+hCG animals (n = 6). Very low signal spots (signal < 50) were removed and normalization to the median array was performed. Unsupervised hierarchical clustering identified two outliers: one sample from an animal with induced disease (induced animal 4; Table 1) in the 6mo+hCG group, clustering well away from the endometriosis group (Fig. 3) and one sample from the DF+hCG group (DF+hCG 6, Fig. 3). These samples were excluded from subsequent analyses.

Class comparison identified 22 and 165 genes whose transcript levels differed more than 2-fold between DF+hCG animals and those in the 3mo+hCG and 6mo+hCG groups, respectively (Supplemental Tables 1 and 2, published on The Endocrine Society's Journals On-

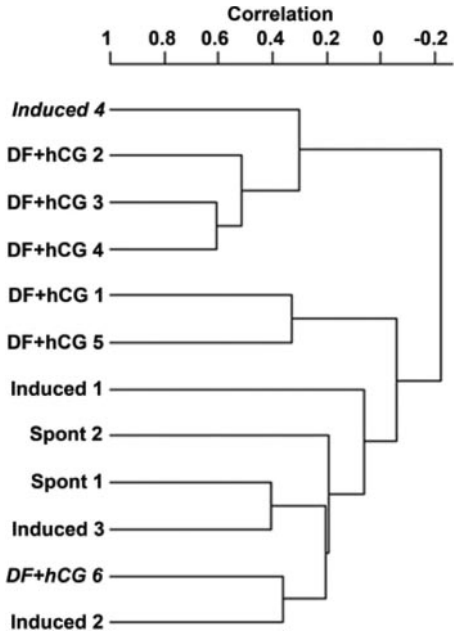

FIG. 3. Dendogram of unsupervised hierarchical clustering of eutopic endometrial gene expression in a model of simulated early pregnancy in baboons with endometriosis. Baboons with experimental and spontaneous endometriosis were treated with hCG during the window of implantation (6mo+hCG). After Illumina Beadchip interrogation of endometrial transcripts from 6mo+hCG animals (n = 6) and DF animals similarly treated with hCG (DF+hCG; n = 6), nonhierarchical clustering was performed using centered correlation and average linkage analysis. Spont1/2, Animals with spontaneous disease; induced 1/2/3/4, animals with experimentally induced disease. Animals in *italics* were considered as outliers and excluded from further analysis.

line web site at http://endo.endojournals.org). Increased levels of 20 and 115 genes were demonstrated at 3 and 6 months of disease, respectively, compared with DF+hCG animals; 14 genes were up-regulated at both time points. Decreased levels of two and 50 genes were evident at 3 and 6 months of disease, respectively; no genes were mutually down-regulated at both times of disease.

Array verification

To validate the microarray analysis, qRT-PCR was used to measure endometrial expression levels of four dysregulated RNA transcripts, namely secreted frizzled-related protein 4 (*SFRP4*), apolipoprotein A1 (*APOA1*), serpin peptidase inhibitor, clade A (*SERPINA3*), and pregnancy-associated plasma protein A (*PAPPA*). qRT-PCR analysis using the same RNA samples as those for the array analysis (DF+hCG, 3mo+hCG, and 6mo+hCG) confirmed a significant 26- and 10-fold increase in endometrial gene expression of *APOA1* in the 3mo+hCG and 6mo+hCG groups, respectively, compared with the DF+hCG group (Fig. 4A; Mann Whitney *U* test, $P < 0.05$). The level of endometrial *SFRP4* expression was significantly increased by 14-fold in the 3mo+hCG and 10-fold in the 6mo+hCG groups compared with the DF+hCG group (Fig. 4B; Mann Whitney *U* test, $P < 0.05$). A significant 4-fold increase in *PAPPA* was demonstrated in endometria from the 3mo+hCG animals, with a continued but nonsignificant increase in the 6mo+hCG group (Fig. 4C). This reveals the novel finding that whereas in DF animals hCG decreases expression of *APOA1*, *SFRP4*, and *PAPPA* compared with untreated controls, in animals with endometriosis, hCG produces the opposite response, increasing their expression. In contrast, for *SERPINA3*, which is increased by hCG treatment in DF animals, the 3mo+hCG and 6mo+hCG animals show a gradual loss in the ability of this transcript to respond to hCG (Fig. 4D; Mann Whitney *U* test, $P < 0.05$).

Immunolocalization of C3 and SOD2

Confirming our previously published data (18), a marked up-regulation of stromal C3 and glandular and stromal SOD2 immunostaining was evident in the endometrial stroma of the DF+hCG group of animals, compared with DF-hCG animals (Fig. 5, B and H, respectively), suggesting a role for C3 and SOD2 in the stromal differentiation that occurs in response to the implanting blastocyst. The induction of C3 protein observed in DF+hCG was markedly attenuated in the 3mo+hCG and 6mo+hCG animals (Fig. 5, C and D). As disease progressed through 12 and 15 months of endometriosis, the induction of C3 protein by hCG was effectively abolished (Fig. 5, E and F). Furthermore, SOD2 protein levels were markedly lower in the endometrium of animals with endometriosis after hCG stimulation, throughout progression of disease compared with DF+hCG animals (Fig. 5, I–L). The failure of hCG to up-regulate C3 and SOD2 protein implies that the differentiation of eutopic endometrium of animals with endometriosis in response to this embryonic signal becomes gradually impaired.

Molecular endometrial response to hCG is altered throughout progression of disease

The molecular endometrial response to hCG was further investigated progressively after induction of endometriosis through 12 and 15 months of disease. We have previously demonstrated down-regulation of *SFRP4* and *APOA1* and up-regulation of *SERPINA3* by hCG in the endometrium of DF animals during the window of implantation (18). As for C3 and SOD2, we sought to confirm that these factors were not only temporarily dysregulated but that hCG failed to regulate their expression throughout progression of disease. Therefore, qRT-PCR for *APOA1*, *SPRP4*, and *SERPINA3* was performed on endometria from DF-hCG animals, DF+hCG animals, and animals with experimentally induced endometriosis, treated with hCG. There was a statistically significant de-

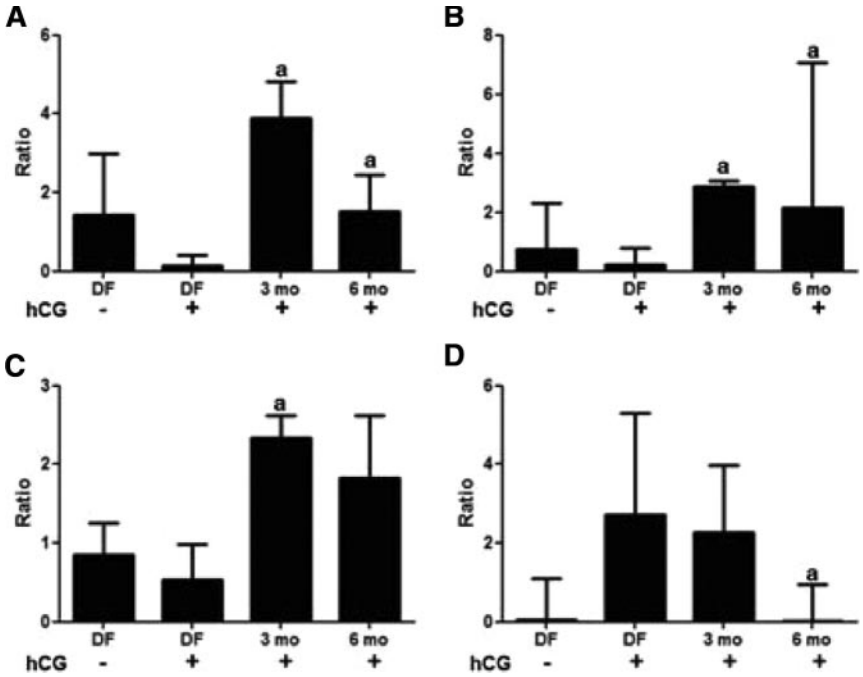

FIG. 4. The eutopic endometrial molecular response to hCG is blunted in baboons with endometriosis. Total endometrial RNA was extracted from baboons with 3 months (n = 4) and 6 months (n = 5) endometriosis and DF animals (n = 5) after hCG infusion during the window of implantation. Additional endometrial RNAs were extracted from unstimulated, DF animals (DF-hCG; n = 6). qRT-PCR analysis revealed that mRNA levels of *APOA1* (A), *SFRP4* (B), and *PAPPA* (C) were significantly increased in both 3mo+hCG (3 mo) and 6mo+hCG (6 mo) animals compared with DF controls (DF+hCG). Conversely, mRNA levels of *SERPINA3* (D) were significantly decreased after hCG infusion in animals with endometriosis compared with DF+hCG control. *Columns* represent the median normalized values against ribosomal 18S. *Error bars*, Interquartile range. a, Significant difference to DF+hCG, $P < 0.05$; Mann-Whitney.

crease in the ability of hCG to down-regulate *APOA1* in animals with endometriosis (Fig. 6A; Kruskal-Wallis ANOVA, $P < 0.05$), thus extending our recently published preliminary observations (26). HCG infusion progressively up-regulated endometrial *SFRP4* mRNA levels throughout progression of disease compared with DF+hCG (Fig. 6B), although this did not quite reach statistical significance (Kruskal-Wallis ANOVA, $P = 0.06$). There was a striking loss in the ability of hCG to induce the expression of *SERPINA3* throughout the progression of endometriosis from 6 through 15 months of disease (Fig. 6C; Kruskal-Wallis ANOVA, $P = 0.05$). No significant difference in *APOA1*, *SFRP4*, or *SERPINA3* mRNA levels was observed when endometria from DF-hCG animals and unstimulated endometriotic animals (1mo-hCG), at the time of cannulation (see Fig. 1), were compared (Mann Whitney *U* test, $P > 0.05$), indicating that the presence of disease itself did not affect levels of these transcripts in unstimulated endometrium not treated with hCG.

Endometrial response to hCG is not a result of altered basal LHCGR distribution

We have previously described that hCG treatment of DF animals during the window of implantation induces a redistribution of endometrial LHCGR from the luminal and glandular epithelium to the stromal cells and cells surrounding the spiral arteries (27). The levels and distribution of LHCGR as assessed by immunostaining are similar in unstimulated animals with endometriosis compared with DF-hCG controls (Fig. 7, E and F). Treatment of DF animals with hCG induced down-regulation of LHCGR within the epithelial compartment as expected (Fig. 7, C and D), but this epithelial response was compromised in animals with endometriosis (Fig. 7G). Interestingly the increased receptor abundance induced by hCG adjacent to the spiral arteries is unaffected (Fig. 7H).

Discussion

Endometriosis is associated with subfertility. It as a matter of intense debate whether the presence of endometriotic foci causes infertility or whether the development of disease is associated with a concurrent inherent reproductive failure. Although multiple factors have been implicated in endometriosis-associated infertility, including both endometrial and ovarian defects, elucidation of its etiology remains elusive because most women present with established disease. In a nonhuman primate model of simulated early pregnancy, with experimentally induced endometriosis, we have now demonstrated an aberrant endometrial response to locally infused hCG, an early embryonic signal. We have previously described morphological and molecular endometrial changes in all three major endometrial cell types, luminal, glandular, and stromal, after *in vivo* infusion of hCG in DF animals (18, 22). These typical changes were not observed in the majority of animals with endometriosis. We are confident that the experimental approach that we have used is robust because using an identical experimental paradigm in DF animals, we have shown that no molecular or cellular endometrial response was observed when heat-inactivated hCG was infused via an oviductal cannula (22), indicating that oviductal manipulation and cannulation has no consequence on endometrial physiology. Also, we have extensively examined gene expression, protein levels, and distribution in the endometrium from control DF and hCG-stimulated and -unstimulated animals (18, 22, 27,

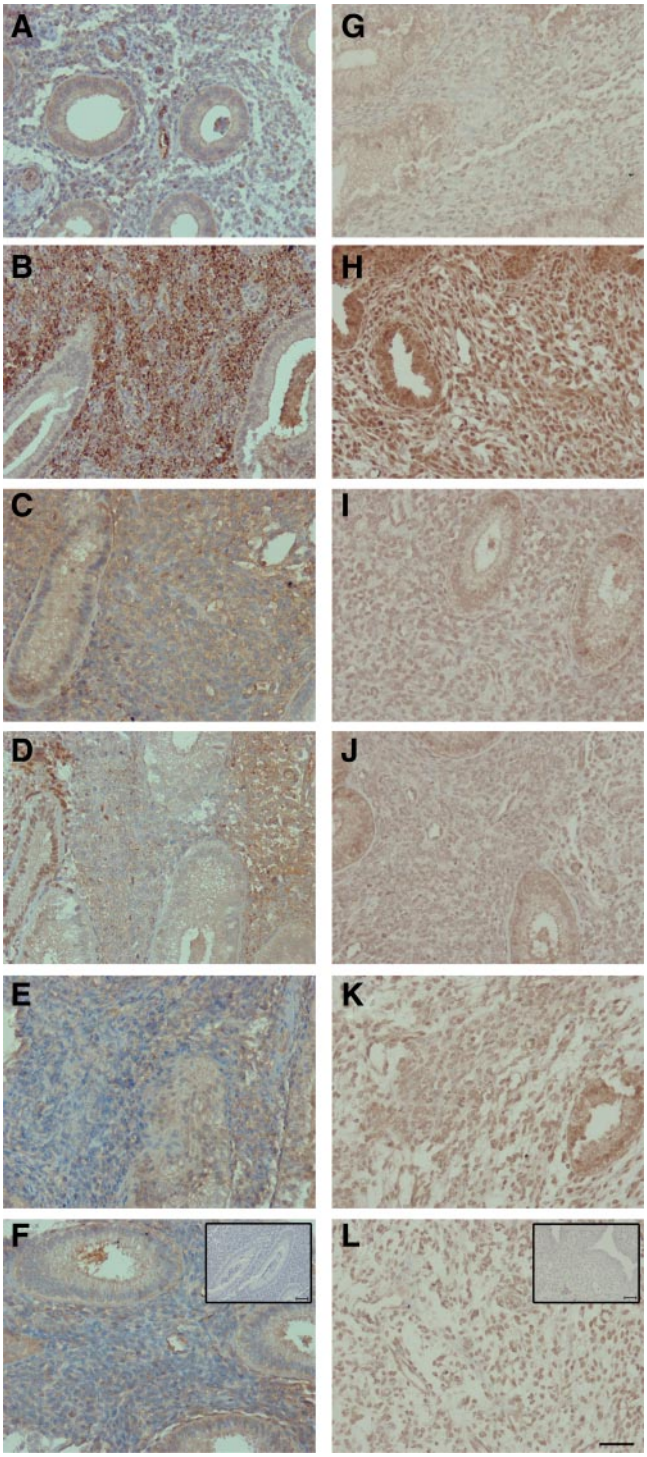

FIG. 5. C3 and SOD2 proteins are down-regulated by hCG in the eutopic endometrium of baboons with endometriosis. The distribution of C3 (*left panel*) and SOD2 (*right panel*) was determined in DF-hCG unstimulated animals, DF animals after hCG infusion (DF+hCG), and throughout the progression of endometriosis from 3 to 12 months of disease. Minimal levels of C3 (A) and SOD2 (G) proteins were detectable in endometria of DF-hCG animals. After hCG infusion in DF+hCG animals, both C3 (B) and SOD2 (H) endometrial protein levels were markedly increased. The ability of hCG to induce C3 and SOD2 was dramatically reduced in animals with endometriosis at 3mo+hCG (C and I), 6mo+hCG (D and J), 12mo+hCG (E and K), and 15mo+hCG (F and L). *Inserts* in F and L represent preimmune controls of C3 and SOD2, respectively. *Bars*, 50 μm.

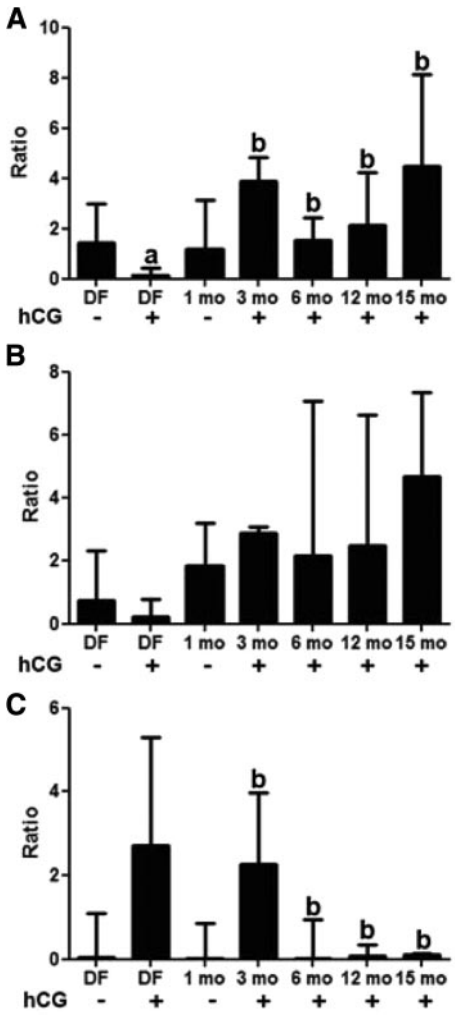

FIG. 6. Molecular dysregulation of the endometrial response to hCG is apparent throughout progression of disease in baboons with endometriosis. Total endometrial RNA was extracted from baboons with 3 months (mo; n = 4), 6 months (n = 5), 12 months (n = 3), and 15 months (n = 2) endometriosis and DF animals (DF+hCG; n = 5) after hCG infusion during the window of implantation. Additional RNAs were extracted from unstimulated DF animals (DF-hCG; n = 6) and unstimulated animals with 1 month endometriosis (n = 4). qRT-PCR analysis revealed a loss of CG-induced down-regulation of endometrial expression of *APOA1* (A) and *SFRP4* (B). In contrast, *SERPINA3* mRNA was up-regulated by hCG in DF animals (C). *Columns* represent medians normalized values against ribosomal 18S. *Error bars*, Interquartile range. a, Significant difference to DF-CG, Mann Whitney *U* test, $P < 0.05$; b, a significant difference to DF+hCG; Kruskal Wallis ANOVA, $P < 0.05$).

28). In these studies consecutive surgical manipulation and/or hCG infusion does not alter the endometrial phenotype.

In addition, we have previously examined global endometrial gene expression in unstimulated baboons with endometriosis, throughout an equivalent time course, identifying dysregulated genes due to the presence of disease, before any modification by blastocyst derived signals (28). When these gene lists were cross-referenced to that of the 3mo+hCG group, four genes were identified as com-

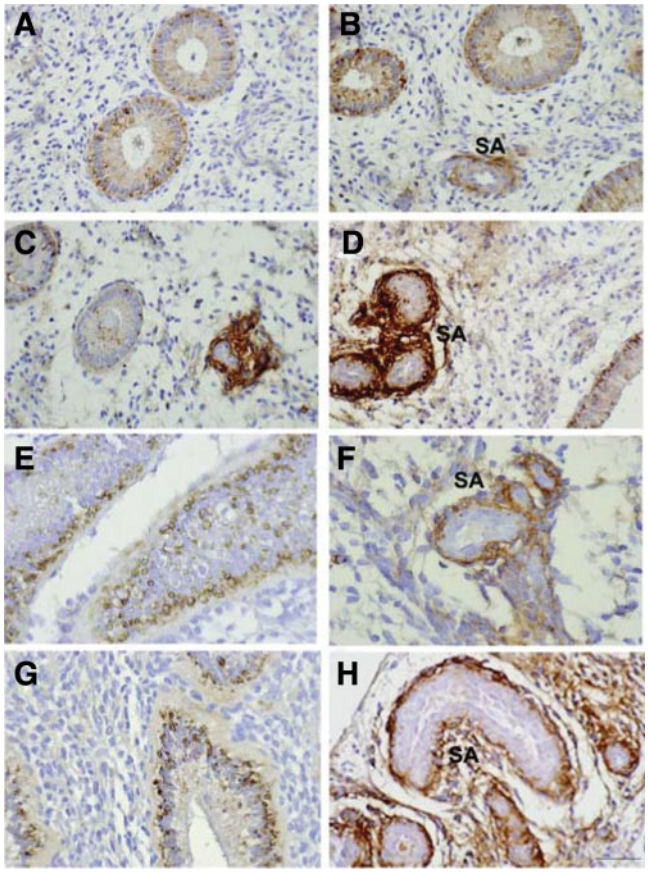

FIG. 7. LHCGR immunostaining of eutopic endometrium from DF and endometriotic animals. Immunohistochemistry was performed using polyclonal antibodies against the extracellular domain of the LHCGR. LHCGR protein was detectable in glandular epithelial cells in the functionalis (A) and basalis (B) endometrium of unstimulated DF baboons (DF-hCG) during the window of implantation. After hCG infusion in DF animals (DF+hCG), glandular LHCGR protein levels reduced (C), whereas increased levels were observed surrounding the spiral arteries, predominantly in the basalis endometrium (D). Normal baseline levels of LHCGR were present in unstimulated functionalis (E) and basalis (F) eutopic endometrium of animals with endometriosis at 1 month of disease. However, the hCG-induced down-regulation of glandular LHCGR in animals with endometriosis was not evident in 6mo+hCG animals (G), whereas the up-regulation adjacent to the spiral arteries remained evident (H). *Bar*, 50 μm. SA, Spiral artery.

mon to both lists [maternally expressed 3 (*MEG3*), Rho GTPase activating protein 20 (*ARHGAP20*), and nephronophthisis (*NPHP4*)], as described in Supplemental Table 1. Aberrant transcript levels of *ARHGAP20*, *MEG3*, and *NPHP4* were also demonstrated in the 6mo+hCG group compared with the DF+hCG group. Indeed, the expression of 38 of the 165 genes that differed between the 6mo+hCG group and DF+hCG animals also differed between DF-hCG animals and those with experimentally induced endometriosis, in the absence of any hCG treatment. Of the 127 genes that failed to respond normally to hCG in animals with 6 months endometriosis, we have previously shown nine to be regulated by this hormone in DF animals during the window of implantation (18). For example, *APOA1* and *SFRP4*, previously shown to be down-regulated by hCG in DF animals, are now up-regulated by hCG in animals with endometriosis. Conversely, *SERPINA3*, which is normally up-regulated by hCG in the absence of any disease, is actively down-regulated by hCG in animals with endometriosis. We confirmed the dysregulation of several of these genes and their translated proteins, not only at the time point of array analysis (3 and 6 months) but also throughout progression of disease (to 12 and 15 months). This longitudinal study revealed that not only are the responses fundamentally altered in the presence of endometriosis but that the response to hCG changes with time as the disease progresses.

This new study has shown that the endometrial response to hCG in animals with endometriosis is compromised in multiple pathways. Many of these transcripts are involved in the induction of a receptive endometrium. hCG induced a statistically significant increase in endometrial *SFRP4* in animals with endometriosis compared with DF controls (Figs. 4 and 6). Secreted frizzled-related proteins are regulators of the Wnt signaling pathway, modulating embryogenesis, cell proliferation, differentiation, adhesion, and apoptosis (29, 30). *SFRP4* expression is decreased in the secretory phase in both human and nonhuman primate endometrium, suggesting down-regulation by progesterone (31, 32). In DF animals, the effect of hCG is to further down-regulate this transcript, indicating that it may be involved in hCG-induced inhibition of apoptosis (33) and the promotion of stromal cell decidualization (18). Although no direct role for *SFRP4* has been reported in the process of decidualization, we propose that the increase in *SFRP4* induced by hCG in animals with endometriosis may alter stromal cell proliferation and thus contribute to defective decidualization. Other factors that show altered responses to hCG in this study, such as the reactive oxygen species scavenger, SOD2 (also known as manganese-SOD), are known to participate in decidualization and could clearly affect endometrial stromal cell differentiation during early pregnancy. SOD2 is up-regulated by hCG in DF animals but is down-regulated by hCG in animals with endometriosis. Sugino *et al.* (34) have demonstrated that *in vitro* decidualization of human endometrial stromal cells resulted in the up-regulation of SOD mRNA and protein activity. Ho *et al.* (35) demonstrated that silencing of SOD resulted in postimplantation pregnancy loss. Together these data suggest that increased reactive oxygen species scavenging is critical for establishment and maintenance of pregnancy. Our data suggest that hCG may play an important role in this process and that endometriosis may dysregulate this response.

hCG down-regulated C3 protein in animals with endometriosis (Fig. 5). Intriguingly, a study from the early

1990s in women showed increased levels of C3 in endometriotic tissues (36). However, our data, specifically addressing the response to hCG, concurs with recent work from Chow *et al.* (37), showing that C3 deficiency impairs early pregnancy in mice. Indeed, we have previously proposed that hCG-induced up-regulation of stromal C3 in DF animals may orchestrate the endometrial immune adaptation to pregnancy (18). We have now demonstrated that not only C3 but also several additional immunoregulatory factors are dysregulated by hCG in the endometrium of baboons with endometriosis, including IL-1 receptor type II and other members of the complement pathway. Altered regulation of these factors by hCG in endometriosis may lead to an unfavorable immunological environment resulting in the rejection of the fetal allograft.

There are a number of possible explanations for the aberrant endometrial response to locally infused hCG in animals with endometriosis. In primary cultures of baboon endometrial epithelial cells, hCG does not activate the classical Gs/adenyl cyclase/cAMP/protein kinase A pathway but induces rapid phosphorylation of ERK 1/2 in a protein kinase A-independent manner (20, 21). Both blood and peritoneal fluid from women with endometriosis contain elevated levels of proinflammatory cytokines (38, 39). These can alter expression of naturally occurring suppressors of cytokine signaling such as suppressor of cytokine signaling-1, which can inhibit ERK 1/2 activation (40). Cytokines and other factors, produced by ectopic endometrial lesions, may therefore inhibit endometrial LHCGR signaling, via the ERK 1/2 pathway. Alternatively, the presence of endometriosis may inhibit the hCG-induced redistribution of LHCGR from the luminal and glandular epithelium to the stromal cells surrounding the spiral arteries that is seen during early pregnancy (22). Whereas studies have revealed conflicting data on LHCGR distribution in unstimulated eutopic endometriotic endometrium (28, 41, 42), our data support the latter hypothesis (Fig. 7).

Decidualization itself is known to regulate LHCGR abundance (27). Abnormal decidualization may therefore cause changes in LHCGR distribution. There is strong evidence that decidualization is defective in women and baboons with endometriosis (6, 7, 43). The level and localization of LHCGR were normal in animals with endometriosis before hCG stimulation (Fig. 7), suggesting that the failure of LHCGR to relocate in response to hCG is caused by other factors. We and others have demonstrated significant down-regulation of the progesterone receptor (PGR) in eutopic endometriotic endometrial epithelium (44–46). We have previously shown in DF animals that the response to hCG could be suppressed by antagonism of the PGR (47). We hypothesize that the altered responses to hCG may be due in part to decreased PGR and the progesterone resistance associated with endometriosis. In support of this, PGR expression is significantly lower at 6 months compared with 3 months in baboons with endometriosis (46). This correlates with the greater degree of gene dysregulation present in animals at 6 months of disease compared with 3 months when stimulated with hCG (Supplemental Tables 1 and 2). Furthermore, whereas there was no change in the stromal cell distribution of PGR, the ability of PGR to respond to hormonal stimuli is diminished in the stromal cells isolated from animals with both induced and spontaneous disease, suggesting complex paracrine interactions between the stromal and epithelial compartments of the endometrium, a paradigm eloquently described by Cooke *et al.* (48). We and others have demonstrated aberrant expression of many progesterone-regulated genes throughout the endometrial cavity in spontaneous and experimental model of endometriosis, including *HOXA10*, calcitonin (*CALC*), and tissue transglutaminase 2 (*TGM2*) (5, 43, 49). Furthermore, emerging evidence supports a widespread progesterone resistance in endometriosis (50), and we have recently demonstrated that the molecular aberrations evident in the eutopic endometrium are also evident in oviductal tissues of animals with endometriosis. These responses were similar to those observed in DF animals treated with PGR antagonist (51).

We have previously noted that a number of genes are coregulated in the endometrium by hCG and progesterone (18). This coregulation may explain why IVF treatment protocols with supraphysiological treatment levels of progesterone can overcome any abnormal endometrial response to hCG that is seen in the presence of endometriosis. An example of this is FK506-binding protein 4 (FKBP52), a cochaperone for the PGR, that stabilizes the PGR complex to optimize progesterone binding and subsequent signaling (52). Null mutants for *FKBP52* show a reduced uterine PGR responsiveness, which causes implantation failure. This can be reversed by supraphysiological levels of progesterone supplementation. Thus, endometrial implantation failure, caused by a deficiency of one component of the progesterone signaling pathway, can be overcome by high levels of progesterone. Interestingly, women and baboons with endometriosis have reduced *FKBP52* expression in eutopic and ectopic endometrium, compared with control eutopic endometrium (32, 53). It is possible that endometrial deficiency in *FKBP52* and other coregulated genes can be overcome by high levels of progesterone. Based on this study, we speculate that a similar effect could occur by the direct action of hCG on the endometrium. Huber *et al.* (54) have also proposed that hCG has therapeutic potential in the suppression of endometriotic foci. High doses of hCG may

therefore enhance receptivity in two ways: first, by synergistically overcoming progesterone resistance directly within the endometrium and second, by diminishing the secretion of proinflammatory cytokines from endometriotic foci. Exogenous hCG may also exert a luteotrophic effect to augment serum progesterone levels, overcoming endometriosis-associated infertility by providing critical luteal support necessary for the establishment and maintenance of pregnancy.

We and others have also shown epigenetic modification of eutopic endometrial gene expression in the presence of endometriosis (43, 55, 56). In particular, we have shown hypermethylation of *HOXA10*, a gene known to be critical for implantation and decidualization (43) and hypomethylation of *WNT2B*, a member of the Wnt signaling family (24). More recently epigenetic regulation of E-cadherin (*CDH1*), an epithelial protein thought to be critical for implantation, has been demonstrated (57). Expression of E-cadherin protein in the eutopic endometrium of baboons with endometriosis is abnormal (49). Therefore, epigenetic transformation of eutopic endometriotic endometrium may render it incapable of responding appropriately to paracrine or autocrine signals, such as hCG. Because DNA hypermethylation is relatively stable, the effect of removing ectopic lesions on eutopic endometrial gene expression and function is unpredictable.

In conclusion, these data suggest that in a simulated model of early pregnancy, the induction of endometriosis results in altered responses to a major embryonic signal, hCG. Some endometrial transcripts, which are normally regulated by hCG, show attenuated responses, whereas others respond in the opposite manner to that seen in normal animals. These altered responses prevent the acquisition of the full endometrial molecular repertoire necessary for implantation. We propose that this attenuated response is, in part, a consequence of the progesterone resistance associated with endometriosis. The fact that this dysregulation is seen only after the induction of endometriosis indicates that it is the presence of the endometriotic lesions that causes the altered endometrial responses to hCG. The changes in animals with induced endometriosis were paralleled in animals with spontaneous disease, validating the experimental model as a powerful tool to understand the pathophysiology of endometriosis-associated infertility. We propose that the reduced fecundity associated with endometriosis has a multifactorial etiology, including abnormalities of differentiation, immunological and decidualization pathways, which ultimately create an endometrial environment that is unreceptive to an implanting blastocyst.

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