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Gene Expression Analysis of Endometrium Reveals Progesterone Resistance and Candidate Susceptibility Genes in Women with Endometriosis

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The identification of molecular differences in the endometrium of women with endometriosis is an important step toward understanding the pathogenesis of this condition and toward developing novel strategies for the treatment of associated infertility and pain. In this study, we conducted global gene expression analysis of endometrium from women with and without moderate/severe stage endometriosis and compared the gene expression signatures across various phases of the menstrual cycle. The transcriptome analysis revealed molecular dysregulation of the proliferative-to-secretory transition in endometrium of women with endometriosis. Paralleled gene expression analysis of endometrial specimens obtained during the early secretory phase demonstrated a signature of enhanced cellular survival and persistent expression of genes involved in DNA synthesis and cellular mitosis in the setting of endometriosis. Comparative gene expression analysis of progesterone-regulated genes in secretory phase endometrium confirmed the observation of attenuated progesterone response. Additionally, interesting candidate susceptibility genes were identified that may be associated with this disorder, including FOXO1A, MIG6, and CYP26A1. Collectively these findings provide a framework for further investigations on causality and mechanisms underlying attenuated progesterone response in endometrium of women with endometriosis. (*Endocrinology* 148: 3814–3826, 2007)

ENDOMETRIOSIS IS A COMPLEX disorder associated with pelvic pain and infertility and is characterized by the implantation of endometrial tissue outside the uterus, primarily on the pelvic peritoneum and ovaries (1). Endometriosis affects 6–10% of women in the general population and 35–50% of women with pain and/or infertility (2). It is widely accepted that by retrograde menstruation (3), endometrial tissue establishes itself on the peritoneum of women with endometriosis due to heritable and/or acquired defects that confer survival advantage and promote attachment, growth, neoangiogenesis, and invasion into the peritoneum.

Although the estrogen dependence of endometriosis is well established, the role of progesterone in this disorder is comparatively less well developed. The relative balance of progesterone and estrogen steroidal activity governs the function of normal endometrium throughout the menstrual cycle. The growth-promoting effects of estrogen during the proliferative phase of the cycle are countered by progesterone's antiproliferative actions at the postovulatory onset of the secretory phase and decidualizing effects on endometrial stroma later in the secretory phase (4, 5). A phenotype of attenuated progesterone response is suggested in endometriosis, and interestingly, progestin-based treatment of pain associated with this disorder is variably effective (6, 7).

We and others (8–13) reported dysregulation of various progesterone target genes during the implantation window in women with endometriosis. An endometrial microenvironment characterized by attenuated progesterone response may be inhospitable to embryonic implantation. Reduced responsiveness, or resistance, to progesterone in eutopic endometrium has been implicated in the pathophysiology of this enigmatic condition, as suggested by the altered pattern of matrix metalloproteinase (MMP) gene expression in the secretory phase (14). Interestingly, *in vitro* treatment of endometrial tissues acquired from women with endometriosis with progesterone fails to fully suppress either pro-MMP-3 or pro-MMP-7 secretion and fails to prevent the ability of these tissues to establish experimental disease in mice (15). More recently, endometrial cell culture and nude mouse models were used to demonstrate that progesterone insensitivity was intrinsic to the eutopic endometrium of women with endometriosis and could be corrected by treatment with the synthetic progestin, tanaproget (16).

Progesterone resistance may occur at the level of the progesterone receptor (PR) isoforms (PR-A and PR-B) (17, 18), steroid receptor coactivators, or downstream effectors (TGFβ, Dickkopf-1, retinoic acid, c-*myc*, *etc.*). In endometri-

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Abbreviations: CYP26A1, Cytochrome P450, family 26, subfamily A, polypeptide 1; EGFR, epidermal growth factor receptor; ESE, early secretory endometrium; GO, gene ontology; IGFBP, IGF binding protein; MCM, minichromosome maintenance; MIG6, mitogen-inducible gene 6; MSE, midsecretory endometrium; MMP, matrix metalloproteinase; MT, metallothionein; PCA, principal component analysis; PE, proliferative endometrium; PR, progesterone receptor; TOB1, transducer of ErbB-2.

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otic lesions, a decrease in the expression of the progesterone target gene, 17-β hydroxysteroid dehydrogenase type I, is evidence of progesterone resistance in ectopic endometrium (19, 20). In the current study, we applied paralleled gene expression analysis to investigate cycle phase-dependent differences in the eutopic endometrial gene expression signatures across the menstrual cycle of women with moderate/severe disease, compared with normals. In women with moderate/severe disease, the gene expression profile suggested incomplete transitioning of the endometrium from the proliferative to early secretory phase, a phenotype of enhanced cellular survival, and attenuation of progesterone-induced down-regulation of DNA synthesis and cellular mitosis. Additionally, the secretory endometrium from women with disease demonstrated dysregulation of numerous genes known to be progesterone regulated. These results provide compelling molecular evidence for attenuated progesterone responsiveness within eutopic endometrium in women with endometriosis.

Materials and Methods

Tissue specimens

All patients provided informed consent for participation in this study under a protocol approved by the University of California, San Francisco, Committee on Human Research and the Stanford University Committee on the Use of Human Subjects in Medical Research. Because endometriosis is a visually heterogeneous condition and studies have documented inaccuracy in its visual diagnosis, particularly in cases of minimal/mild stages (21, 22), we sought to improve the fidelity of our study cohort by including only women with surgically documented and histologically validated moderate/severe-stage endometriosis. Accordingly, endometrial biopsies were obtained from normally cycling women with histologically confirmed, moderate-severe endometriosis at laparoscopy (n = 21) and normally cycling women found to be free of endometriosis at surgery (n = 16). Moderate to severe endometriosis (stage III-IV disease) was defined in accordance with the revised American Fertility Society classification system (23). Study subjects in the severe endometriosis cohort were 22–44 yr old, had regular menstrual cycles, and were documented not to be pregnant at the time of surgery. Many of these patients were also infertile and several had failed *in vitro* fertilization treatment(s) before laparoscopic surgery (24). The demographic profile of the endometriosis-free cohort has been described previously (25). The demographic profile of the cohort with endometriosis is provided in Table 1. Subjects using any form of hormonal treatment within 3 months of biopsy were excluded from the study. Biopsy specimens were obtained using either Pipelle catheters or curette from the uterine fundus under sterile conditions. In comparable subjects without endometriosis, we reported minimal variation between these sampling methods when comparing endometrial molecular profiles (25). Samples were processed for histologic confirmation as well as for RNA isolation. Endometrium was dated by up to four independent histopathologists, all of whom were blinded to the subject's identity and timing of the biopsy. Histological dating was based on the method of Noyes *et al.* (26). Specimens were classified as proliferative (PE, d 8–14), early secretory (ESE, d 15–18), midsecretory (MSE, d 19–23), or late secretory (d 24–28) endometrium.

RNA preparation/target preparation/array hybridization and scanning

A total of 37 specimens were used for microarray analysis, with 21 specimens (PE = 6, ESE = 6, MSE = 9) obtained from subjects surgically confirmed to be affected by moderate-severe endometriosis and 16 specimens (PE = 5, ESE = 3, MSE = 8) obtained from subjects surgically confirmed to be free of endometriosis. The latter samples were used previously to define the normal endometrial expression signature across the various phases of the menstrual cycle (25). Each endometrial biopsy specimen was processed individually for microarray hybridization. Briefly, total RNA was extracted from each whole-tissue specimen using Trizol reagent (Invitrogen, Carlsbad, CA), subjected to DNase treatment, and purified using the RNeasy Kit (QIAGEN, Valencia, CA). RNA quality was confirmed by A260/A280 ratio and agarose gel electrophoresis, during which resolution of distinct 28s and 18s rRNA bands was used to suppose intact RNA. Using 5 μg of template, double-stranded cDNA and biotinylated cRNA were prepared by methods previously described (25). After chemical fragmentation with 5× fragmentation buffer [200 mM Tris (pH 8.1), 500 mM KOAc, 150 mM MgOAc], biotinylated cRNAs were hybridized to an HU133 Plus 2.0 version high-density oligonucleotide array (Affymetrix, Santa Clara, CA) on an

TABLE 1. Subject characteristics: moderate/severe endometriosis cohort (n = 21)

Patient ID	Cycle phase	Age (yr)	Distribution	Diagnoses	Ethnicity	Medications
26A	Pro	31	O, PI		Caucasian	
587	Pro	37	PI	Liver endo	Caucasian	
647	Pro	39	R, O, PI	Leiomyoma	Caucasian	
594	Pro	38	O, PI		Caucasian	Amour thyroid
651	Pro	37	O, PI	Leiomyoma	Caucasian	Advair, rhinocort
508	Pro	25	R, O, PI		Caucasian	Atenolol
489	ES	39	R, PI	Leiomyoma	Asian	Levothyroxine
496	ES	37	O, PI	Leiomyoma	Caucasian	Advair, rhinocort
599	ES	35	R, O, PI		Black	
27A	ES	22	R, O, PI		Caucasian	
517	ES	35	R, PI	Leiomyoma	Asian	Trental, ciprofloxacin
575	ES	26	O, PI		Unknown	
33A	MS	27	R, PI		Caucasian	
7A/97A	MS	35	O, PI		Unknown	
73A	MS	26	O, PI		Caucasian	
516	MS	34	R, PI	Leiomyoma	Asian	
540	MS	37	R, PI		Caucasian	Keflex prn
543	MS	38	R, O, PI		Caucasian	
678	MS	44	R, PI	Leiomyoma	Asian	
72A	MS	31	PI		Caucasian	
645	MS	39	R, PI		Asian Indian	

All endometrial specimens were taken from subjects surgically staged with moderate/severe endometriosis in accordance with rAFS criteria (23). Pro, Proliferative; ES, early secretory; MS, midsecretory; PI, peritoneal endometriosis, defined as biopsy-proven serosal implant; O, ovarian endometriosis, defined as biopsy-proven endometrioma; R, rectovaginal endometriosis, defined as posterior cul de sac obliteration due to endometriotic lesions; endo, endometriosis; prn, as needed.

Affymetrix fluidics station at the Stanford University School of Medicine Protein and Nucleic Acid facility. Fluorescent labeling of samples and laser confocal scanning of the arrays were conducted at the Protein and Nucleic Acid facility.

Microarray gene expression data analysis

The data generated by the Affymetrix GeneChip Operating Software analysis of the scanned array images were imported into GeneSpring version 7.2 (Agilent Technologies Inc., Santa Clara, CA) for analysis. The data files containing the probe level intensities were processed using the robust microarray analysis algorithm (GeneSpring) for background adjustment, normalization, and log2 transformation of perfect match values (9). Per-chip and per-gene normalization were conducted using GeneSpring normalization algorithms. The normalized data were used in pairwise comparisons of cycle phase-specific endometrium from subjects with and without moderate-severe endometriosis. The resulting gene lists from each pairwise comparison included only the genes that evidenced a fold change of 1.5 or higher and a $P < 0.05$ by a one-way ANOVA parametric test and a Benjamini-Hochberg multiple testing correction for false discovery rate, as described (25). To identify samples with similar patterns of gene expression, principal component analysis (PCA) was performed in which a multidimensional data set is displayed in reduced dimensionality, with each dimension representing a component to which a certain percentage of variance in the data are attributed. The PCA algorithm in GeneSpring was applied to all endometrial specimens grouped by disease status and cycle phase using all 54,600 genes and expressed sequence tags on the HG U133 Plus 2.0 chip to evaluate for similar gene expression patterns and underlying cluster structures, as described (25). To further evaluate for patterns in the gene expression profiles, hierarchical clustering analysis of the combined (pairwise comparisons derived) gene list and all samples was conducted using the smooth correlation for distance measure algorithm (GeneSpring). A Heatmap was generated, which graphically depicts the measured intensity values of the genes, and the dendrogram illustrates relationships between the specimens (25). Raw data files of this experiment are stored at the National Center for Biotechnology Information Gene Expression Omnibus database under the identifier GSE6364.

Gene ontology classification of differentially expressed genes

The integration of gene expression data with the gene ontology was carried out using the gene ontology (GO) tree machine (27). The GO tree machine builds significant biological processes, molecular functions, and cellular components in a gene list as previously described (14).

Validation of microarray data by real-time PCR

Genes of different expression fold changes in each menstrual cycle phase were selected for validation by real-time PCR as described previously (25). Real-time PCR was performed on a minimum of n = 3 samples in both the normal and disease conditions for the proliferative, early secretory, and midsecretory phases. First-strand cDNA was generated from 1 μg total RNA using the Omniscript RT kit (QIAGEN). PCRs were performed in triplicate in 25 μl using the Brilliant SYBR Green PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. Ribosomal protein L19 was chosen for use as a normalizer due to the low variation in expression levels evidenced by this gene in the microarray data set. Intron-spanning PCR primers were designed for each gene of interest (Table 2). Data analysis of the real-time PCR data was conducted as described previously (25). We considered the normal endometrial specimens as control samples and the endometrial specimens from subjects with severe endometriosis as our test samples when conducting fold change calculations from the raw threshold cycle values. Statistical analysis of the PCR data was conducted using the relative expression software tool algorithm, which uses a pairwise-fixed reallocation and randomization test to determine significance (28).

Results

Cluster analysis

PCA revealed that endometrial samples from subjects with endometriosis cluster by cycle phase with samples from subjects without disease (Fig. 1). PCA depicts the variance in gene expression profiles among specimens. For purposes of comparison, samples were grouped by cycle phase. On the three-dimensional graphic, the distance between two plotted points is proportional to the degree of similarity between the two groups' gene expression profiles, using all of the genes and expressed sequence tags on the Affymetrix gene chip HG 133 Plus 2.0. Clustering was more dependent on cycle phase than endometriosis status. The largest variance between specimens from subjects with and without moderate/severe endometriosis was observed in the early secretory phase. Interestingly, the ESE specimens from women with endo-

TABLE 2. Primer sequences used in real-time PCR reactions

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Unigene ID	Amp. (bp)
S100A8	CAGCTGTCTTTCAGAAGACCTG	TGAGGACACTCGGTCTCTAGC	Hs.416073	153
SUI1	ATTGAGCATCCGGAATATGG	TGATCGTCCTTAGCCAGTCC	Hs.150580	101
LTF	GACTCCATGGCAAAACAACA	GAGGAATTCACAGGCTTCCA	Hs.437457	121
IHH	CGGCTTTGACTGGGTGTATT	GAAAATGAGCACATCGCTGA	Hs.115274	217
Patched	TCGAAGGTGGAAGTCATTGAG	CACAGGGCATCTTTTCCATAA	Hs.159526	184
OVGP1	TATGTCCCGTATGCCAACAA	ACGTAGACAAGGGGGAAAGG	Hs.1154	253
TOP2A	AAGCCCTCCTGCTACACATTT	CAGGCTTTTGAGAGACACCAG	Hs.156346	191
CDK1	GCTTATGCAGGATTCCAGGTT	CAATCCCCTGTAGGATTTGGT	Hs.334562	143
FLJ10540	CTCAAGACCGTTGTCTCTTCG	TTCCCACTTGTGATTTCATCC	Hs.14559	197
MT1H	GCAAGTGCAAAAAGTGCAAAT	CACTTCTCTGACGCCCCTTT	Hs.438462	115
SCGB2A2	ACCATGAAGTTGCTGATGGTC	GGCATTTGTAGTGGCATTGTC	Hs.46452	177
CYP26A1	GCATCGAGCAGAACATTCG	TGGAGAACATGTGGGTAGAGC	Hs.150595	235
PSD	AGCTCCCAAAAGAAGTTCAGC	ACTCCAGGTAGGCCTCCTTCT	Hs.154658	199
SH3D5	CCACAGAATGATGATGAGTTGG	GTTGCCTGGAAAAGTACCAAA	Hs.108924	126
TACC2	AGGAGAGCCCTGTCAAGTCAT	CTTCTGGGAGGATTTCTCTGG	Hs.23196	185
SEMA3C	AAGTCTCCGCAGGCATCTATC	CAACAGCCACCATTTCTGAAT	Hs.171921	226
BIRC5	CACTGAGAACGAGCCAGACTT	AACCGGACGAATGCTTTTTAT	Hs.1578	110
ERRFI1/MIG6	TTGCTGCTCAGGAGATCAGA	TTCAGACTGTAGGCCATGGTT	Hs.11169	154
ERBB2	CCCTGGTCACCTACAACACAG	CTCTGCTGTCACCTCTTGGTT	Hs.446352	167
FOXO1	AAGAGCGTGCCCTACTTCAA	CTGTTGTTGTCCATGGATGC	Hs.170133	209
PLZF	CCACCCCTACGAGTGTGAGT	GCTTGATCATGGCCGAGTAG	Hs.591945	230
SPP1	AGAAGTTTCGCAGACCTGACA	GTCATCCAGCTGACTCGTTTC	Hs.313	182

Amp., Amplicon.

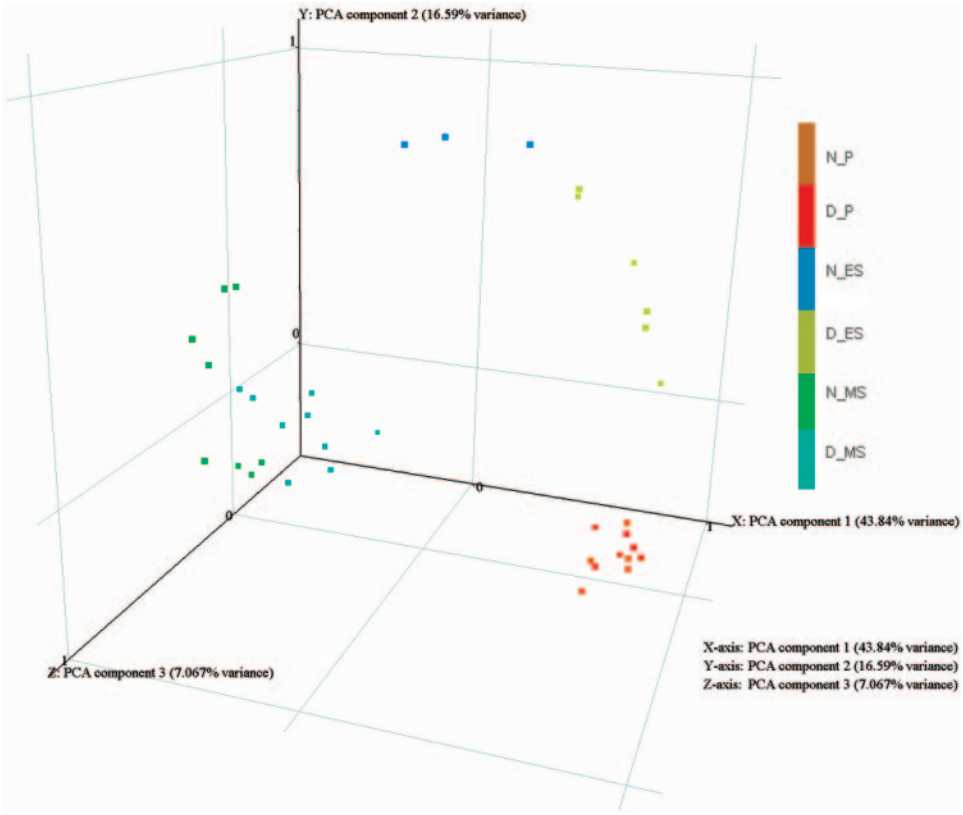

FIG. 1. PCA of endometrium from subjects with moderate/severe endometriosis (D) and subjects without disease (N) in the proliferative (P), early secretory (ES), and midsecretory (MS) phases. Each *plotted point* represents an individual sample's expression profile distributed into a three-dimensional space based on the variance in gene expression. The *labeled axes* represent three PCA components and the percentage is the amount of gene expression variation (in the entire data set) explained by each component.

metriosis collectively plotted much closer to the PE specimens than did the normal ESE specimens, suggestive of attenuation of the progesterone mediated transition on the molecular level.

Unsupervised hierarchical clustering analysis was conducted using the gene expression profiles of the 37 endometrial samples (21 with endometriosis, 16 without endometriosis) based on the combined list of genes showing differential expression throughout the comparable phases of the menstrual cycle. As evidenced by the dendrogram of sample clustering (Fig. 2), the samples self-segregate according to cycle phase, confirming our previously reported observation of phase-dependent segregation of endometrial samples (25). Additionally, within the early secretory cycle phase, the samples demonstrate striking self-segregation into normal and disease clusters. Three endometrial specimens sampled from patients with endometriosis (599, 517, and 27A) were classified as late proliferative phase by the criteria by Noyes *et al.* (26), mostly on the basis of an increased number of mitotic figures observed in these histologic preparations. However, each specimen's overall gene expression profile clustered with the early secretory phase specimens. Dating of these specimens based on last menstrual period placed them collectively between cycle d 15 and 17, confirming their molecular-based dating in the early secretory phase. To further clarify the dating of these specimens, microarray analysis was conducted comparing these three specimens with the other three ESE specimens (489, 496, and 575). This subanalysis showed no significant differences, thereby validating their correct classification as early secretory.

Expression profiling reveals persistent expression of genes involved in cellular proliferation in ESE from women with endometriosis

Of the three phases of the menstrual cycle investigated, the early secretory phase involved the greatest number of statistically significant and differentially expressed genes in endometrium from women with *vs.* without endometriosis (Table 3). The most highly up- and down-regulated genes are shown in Table 4. The complete gene lists for all cycle phases in women with disease *vs.* normals are provided in supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org. The data have been submitted to the Gene Expression Omnibus database under the identifier GSE6364. The GOs enriched in the ESE of women with endometriosis are mostly involved with mitosis and cell proliferation, processes that, in women without disease, are normally down-regulated in ESE (and up-regulated in PE). The complete GO categories for all phases are provided in supplemental Table 2.

To further define the observation of a persistent cellular proliferation signature in ESE from women with endometriosis, we examined expression of individual genes mapped in the KEGG cell cycle pathway [see Fig. 4 and supplemental Table 3 for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for all phases]. As demonstrated, multiple genes involved in mitotic cell cycle regulation are differentially expressed. The finding of a coherent pattern of consistent dysregulation among multiple genes involved in a pathway or

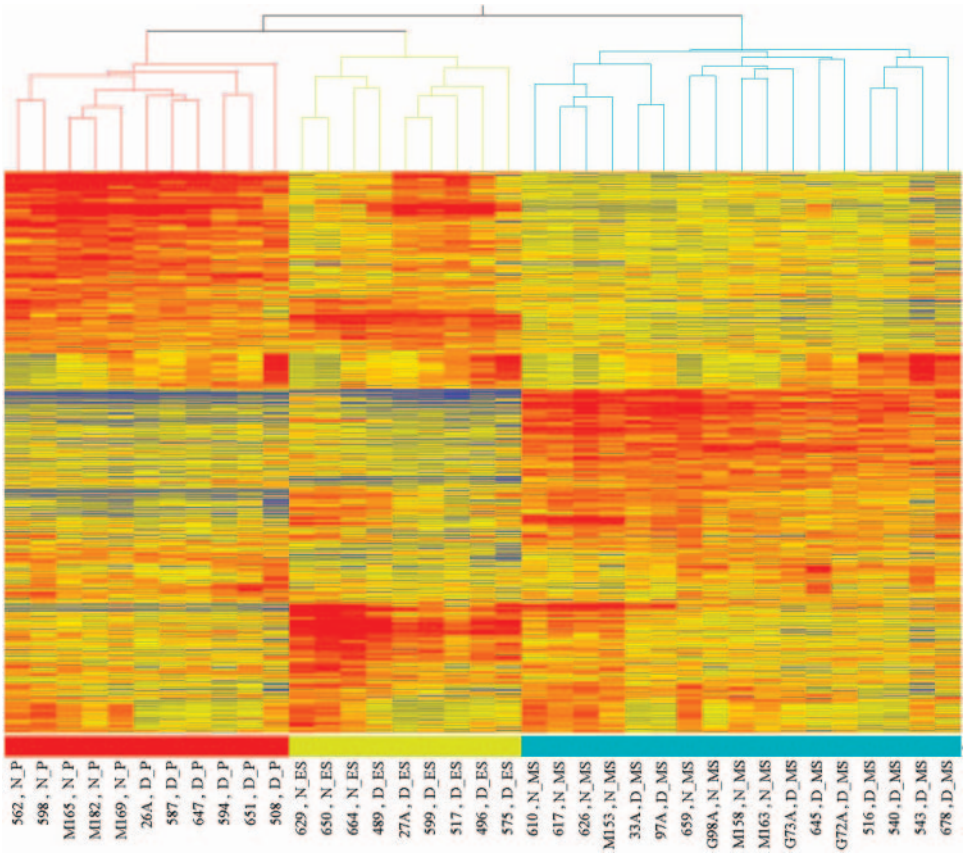

FIG. 2. Hierarchical clustering analysis of endometrium from subjects with moderate/severe endometriosis (D) and subjects without disease (N) in the P (*red*), ES (*gold*), and MS (*light blue*) phases.

process improves the robustness of the finding. Importantly, GO analysis of differentially expressed genes in the proliferative phase did not demonstrate enrichment for genes involved in the cell cycle in endometrium from women with endometriosis. Therefore, the finding of a proliferative gene expression profile persisting in ESE of these women is consistent with reduced progesterone-mediated inhibition of estrogen-induced cellular mitosis.

Progesterone-regulated genes in ESE and MSE from women with vs. without endometriosis

We investigated genes known to be progesterone regulated for dysregulation during the secretory phase in the endometrium from women with endometriosis. Progesterone-regulated genes were identified by systematic review of the literature using the PubMed search engine and were compared against our data set of differentially expressed genes in the secretory phases among women with *vs.* without endometriosis. This approach revealed 54 and 16 dysregulated genes in the ESE and MSE, respectively (Table 5).

TABLE 3. Number of significantly differentially expressed genes in endometrium of endometriosis *vs.* normal subjects at indicated fold change thresholds

	×1.5		×2.0		×4.0	
Menstrual phase	Up	Down	Up	Down	Up	Down
Proliferative	252	447	24	14	2	0
Early secretory	747	1741	213	521	26	59
Midsecretory	428	293	4	22	0	0

Comparison of moderate/severe endometriosis vs. normal and minimal/mild endometriosis vs. normal data sets

We compared the list of differentially expressed genes during the midsecretory phase identified in the current study with the gene list we previously obtained in a comparison of endometrial gene expression profiles during the implantation window in women with *vs.* without minimal/mild endometriosis (8). The two data sets shared five up-regulated genes and 12 down-regulated genes of 1.5-fold or greater (Table 6). Four of the five up-regulated genes are involved in the immune (GZMA, C4BPA) or inflammatory (S100A8, S100A9) responses.

Differentially expressed genes in the region of a locus showing linkage with endometriosis in a genomewide linkage analysis

Recently Treloar *et al.* (29) published the results of a genome-wide linkage analysis study involving 1176 families with affected sibling pairs. This effort identified a region of significant linkage to endometriosis on chromosome 10q26. We searched the genome for genes that fell within the 95% confidence interval of this 10q26 locus and compared these against our data set of differentially expressed genes in the endometrium of women with endometriosis relative to normal endometrium. This analysis identified the following four genes (fold change in endometriosis *vs.* normal endometrium for indicated cycle phase): transforming, acidic coiled-coil containing protein 2 (TACC2; 10q26; −2.86 ESE, −1.59 MSE), a disintegrin and metalloproteinase domain 12 (ADAM12;

TABLE 4. Most highly up- and down-regulated genes per cycle phase-dependent comparison

Gene symbol	Description	Unigene ID	Fold change	*P* value
Proliferative phase				
S100A8	S100 calcium binding protein A8	Hs.416073	4.95	0.0111
SUI1	Putative translation initiation factor	Hs.150580	3.74	0.0136
LTF	Lactotransferrin	Hs.437457	3.51	0.0357
GRAP	GRB2-related adaptor protein	Hs.331099	2.90	0.0125
CD163	CD163 antigen	Hs.74076	2.63	0.0038
DEAD/H	DEAD (Asp-Glu-Ala-Asp) box polypeptide	Hs.349121	−2.63	0.0015
ORM2	Orosomucoid 2	Hs.278388	−2.56	0.0383
PGR	Progesterone receptor	Hs.146046	−2.33	0.0235
IHH	Indian hedgehog homolog	Hs.115274	−2.33	0.0290
OVGP1	Oviductal glycoprotein 1	Hs.1154	−2.32	0.0362
Early secretory phase				
TOP2A	Topoisomerase (DNA) IIα, 170 kDa	Hs.156346	7.59	0.0003
RAB6KIFL	RAB6 interacting, kinesin-like	Hs.73625	5.19	0.0014
APOBEC3B	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	Hs.226307	5.12	0.0011
PENK	Proenkephalin	Hs.339831	5.08	<0.05
TOPK	T-LAK cell-originated protein kinase	Hs.104741	5.05	0.0021
MT1Y	Metallothionein 1Y	Hs.188518	−11.1	0.0079
SCYB13	CXCL13: chemokine ligand 13 (B-cell chemoattractant)	Hs.100431	−9.09	0.0018
CYP26A1	Cytochrome P450, subfamily XXVIA	Hs.150595	−8.33	0.0306
SCGB2A2	Secretoglobin, family 2A, member 2	Hs.46452	−7.69	0.0203
MT1G	Metallothionein 1G	Hs.433391	−7.14	0.0054
Midsecretory phase				
S100A8	S100 calcium binding protein A8	Hs.416073	2.11	0.0156
BLTR2	Leukotriene B4 receptor BLTR2	Hs.130685	2.02	0.0005
MAPK4	Mitogen-activated protein kinase 4	Hs.221703	1.98	0.00001
PTAFR	Platelet-activating factor receptor	Hs.46	1.91	0.0020
GZMA	Granzyme A	Hs.90708	1.87	0.0286
SCGB2A2	Secretoglobin, family 2A, member 2	Hs.46452	−3.33	0.0447
MMP26	Matrix metalloproteinase 26	Hs.204732	−3.03	0.0457
CYP26A1	Cytochrome P450, subfamily XXVIA	Hs.150595	−2.63	0.0306
POMZP3	POM (POM121 homolog, rat) and ZP3 fusion	Hs.296380	−2.56	0.0019
DEPP/C10orf10	Chromosome 10 open reading frame 10	Hs.93675	−2.38	0.0129

Fold changes provided compare the endometrium from women with *vs.* without moderate/severe endometriosis.

10q26.3; 2.29 ESE), arginyltransferase 1 (ATE1; 10q26.13; 1.61 PE, 1.57 ESE), and fibronectin type III and ankyrin repeat domains 1 (FANK1; 10q26.2; −1.85 ESE). Other genes of interest near the 10q26 locus include cytochrome P450, family 26, subfamily A, polypeptide 1 (CYP26A1; 10q23–24; −8.33 ESE, −2.63 MSE); retinol binding protein 4 (RBP4; 10q23–24; −1.72 MSE); pleckstrin and Sec7 domain protein (PSD; 10q24; 2.10 ESE, 1.73 MSE); and sorbin and SH3 domain containing 1 (SORBS1; 10q23–24; 1.61 MSE).

Real-time PCR validation of microarray data

Three up-regulated and three down-regulated genes in each cycle phase comparison (of moderate/severe disease *vs.* normals) that showed statistical significance in the microarray data set were chosen for validation by real-time PCR (Fig. 3). All the genes regulated by microarray in the proliferative phase were confirmed to demonstrate statistically significant regulation in the same direction by real-time PCR (100% concordance). For the early secretory phase, all genes selected for validation exemplified similar direction of regulation to the microarray data, of which five were statistically significant for a concordance of 83%. The exception was CYP26A1, which showed a fold change in the real-time PCR analysis that did not reach statistical significance ($P = 0.097$). In the midsecretory phase, five of the six genes selected for validation showed similar directional change, and four of these achieved statistical significance for a concordance of 67%. The overall concordance rate of significantly regulated genes between the microarray data and the real-time PCR data was 83% (15 of 18).

Discussion

Maintenance of a proliferative fingerprint in the ESE from women with endometriosis

We observed striking enrichment of genes involved in mitosis and proliferation in early secretory endometrium of women with endometriosis (Fig. 4), exceptional insofar as these processes are normally down-regulated in this phase of the cycle and up-regulated during the proliferative phase (25). The finding of enrichment of genes involved in cell cycle regulation was consistent among all ESE specimens from subjects with endometriosis, including those specimens demonstrating concordance for ESE assignment by both histological and molecular dating. Although the overall molecular signature is consistent with the early secretory phase, the genes involved in cell proliferation maintain a fingerprint more consistent with the proliferative phase. A recent study of gene and protein expression in murine luminal epithelium provided evidence for direct inhibition by progesterone of estrogen-induced DNA synthesis in the cell cycle (30). This study showed progesterone down-regulated more than 20 genes associated with DNA replication, most notably the

TABLE 5. Genes previously shown to be progesterone regulated that are dysregulated in endometrium of subjects with moderate/severe endometriosis

Gene symbol	Description	Evidence for progesterone regulation	Unigene ID	Fold change	*P* value
A. Early secretory phase (n = 54)					
MT1Y	Metallothionein 1Y	(25)	Hs.188518	−11.1	0.0079
CYP26A1a	Cytochrome P450, subfamily XXVIA	(54)	Hs.150595	−8.33	0.0306
SCGB2A2	Secretoglobin, family 2A, member 2	(25, 73)	Hs.46452	−7.69	0.0203
MT1G	Metallothionein 1G	(25, 73, 74)	Hs.433391	−7.14	0.0054
MT1X	Metallothionein 1X	(25, 75)	Hs.374950	−7.14	0.0046
MT1F	Metallothionein 1F	(25)	Hs.438737	−6.67	0.0073
CAPN6	Calpain 6	(25, 54)	Hs.169172	−6.25	0.0018
MT1H	Metallothionein 1H	(25)	Hs.438462	−5.56	0.0092
SPP1	Secreted phosphoprotein 1, osteopontin	(25, 74)	Hs.313	−5.56	0.0154
MT1E	Metallothionein 2A	(25, 73–75)	Hs.418241	−4.55	0.0157
ISG20	Interferon stimulated gene, 20 kDa	(54)	Hs.105434	−3.85	0.0008
MAOA	Monoamine oxidase A	(25, 74, 76)	Hs.183109	−3.03	0.0022
GPX3	Glutathione peroxidase 3	(74)	Hs.386793	−2.78	0.0172
MIG6/ERRFI1	Mitogen-inducible gene 6	(77)	Hs.11169	−2.70	0.0025
SGK	Serum/glucocorticoid regulated kinase	(54, 75)	Hs.296323	−2.63	0.0296
DEPP/C10orf10	Chromosome 10 open reading frame 10	(75, 78)	Hs.93675	−2.50	0.0234
DKK1	Dickkopf homolog 1	(8, 25, 74, 75, 79)	Hs.40499	−2.44	<0.05
MUC1	Mucin 1, transmembrane	(25)	Hs.89603	−2.38	0.0094
PAEP	Progestagen-associated endometrial protein/glycodelin	(80–82)	Hs.82269	−2.33	<0.05
FOXO1A	Forkhead box O1A	(25, 83)	Hs.170133	−2.27	0.016
STC1	Stanniocalcin 1	(84)	Hs.25590	−2.08	0.0476
BCAT1	Branched-chain aminotransferase 1	(54)	Hs.438993	−2.00	0.0467
BCL6	B-cell CLL/lymphoma 6	(75)	Hs.155024	−2.00	0.0024
ALDH1A3	Aldehyde dehydrogenase 1 family, A3	(75)	Hs.75746	−1.96	<0.05
NFIL3	Nuclear factor, IL-3 regulated	(54)	Hs.79334	−1.92	0.0167
CITED2	Cbp/p300-interacting transactivator	(54)	Hs.82071	−1.85	0.0097
SAT	Spermidine acetyltransferase 2	(73, 74, 85)	Hs.28491	−1.85	0.0063
RGC32	RGC32 protein	(75)	Hs.76640	−1.82	0.0171
G0S2	Putative lymphocyte G0/G1 switch gene	(75)	Hs.432132	−1.79	0.0441
PTGER2	Prostaglandin E receptor 2 (subtype EP2)	(75)	Hs.2090	−1.79	7.87E-06
PFKFB3	6-Phosphofructo-2-kinase/ fructose-2,6-biphosphatase 3	(54)	Hs.195471	−1.75	0.0156
PPAP2B	Phosphatidic acid phosphatase, type 2B	(54)	Hs.432840	−1.69	0.0028
IRS2	Insulin receptor substrate 2	(75)	Hs.143648	−1.67	0.019
ELL2	Elongation factor, RNA polymerase II	(75)	Hs.173334	−1.64	0.045
SLC2A3	Solute carrier family 2, member 3	(54)	Hs.401274	−1.61	0.0024
PLCB4	Phospholipase C, β4	(75)	Hs.151408	−1.61	0.0177
REV3L	REV3-like, catalytic subunit of DNA polymerase-zeta	(75)	Hs.384997	−1.61	<0.05
IL1R1	IL-1 receptor, type I	(75)	Hs.82112	−1.59	0.011
ATP1B1	ATPase, Na^+/K^+ transporting, β-1	(54)	Hs.78629	−1.58	0.0477
TGFB2	TGFβ2	(86, 87)	Hs.512517	−1.52	0.0144
PENK	Proenkephalin	(25, 73, 74, 88, 89)	Hs.339831	5.08	<0.05
SFRP4	Secreted frizzled-related protein 4	(73, 74, 89, 90)	Hs.105700	4.94	0.0431
MMP11	MMP 11	(25, 73, 74, 89, 91)	Hs.143751	4.02	<0.05
OLFM1	Olfactomedin 1	(25)	Hs.74376	3.45	0.0169
TGFBI	TGF, β-induced	(54, 73, 74, 89)	Hs.421496	3.14	0.0137
TK1	Thymidine kinase 1, soluble	(73)	Hs.164457	2.50	0.0005
MEST	Mesoderm specific transcript homolog	(75)	Hs.440459	2.43	<0.05
THY1	Thy1 cell surface antigen	(25, 73)	Hs.134643	2.35	0.0231
RRM1	Ribonucleotide reductase M1 polypeptide	(73)	Hs.383396	2.25	0.0364
HMGA2	High-mobility group box 2	(54)	Hs.434953	2.15	0.0020
PGR	Progesterone receptor	(35, 36)	Hs.2905	2.12	0.0322
FBN1	Fibrillin 1	(73)	Hs.750	2.03	0.0404
BCL2	B-cell CLL/lymphoma 2	(54)	Hs.79241	1.72	0.0321
MARCKS	Myristoylated alanine-rich protein kinase C substrate	(54, 73)	Hs.318603	1.52	0.0552
B. Midsecretory phase (n = 16)					
SCGB2A2	Secretoglobin, family 2A, member 2	(25, 73)	Hs.46452	−3.33	0.0447
CYP26A1	Cytochrome P450, subfamily XXVIA	(25, 54)	Hs.150595	−2.63	0.0306
DEPP/C10orf10	Chromosome 10 open reading frame 10	(75, 78)	Hs.93675	−2.38	0.0129
SLC15A2	Solute carrier family 15, member 2	(73)	Hs.118747	−1.92	0.0107
IGFBP1	IGFBP 1	(92)	Hs.401316	−1.85	<0.05
ATP1B1	ATPase, Na^+/K^+ transporting, β-1	(54)	Hs.78629	−1.85	<0.05
ELL2	Elongation factor, RNA polymerase II	(75)	Hs.173334	−1.69	0.0461
MT1Y	Metallothionein 1Y	(25)	Hs.188518	−1.67	<0.05
CAPN6	Calpain 6	(25, 54)	Hs.169172	−1.64	<0.05
ENPP1	Ectonucleotide pyrophosphatase/ phosphodiesterase 1	(54)	Hs.213840	−1.59	0.0479

(continues)

TABLE 5. *Continued*

Gene symbol	Description	Evidence for progesterone regulation	Unigene ID	Fold change	*P* value
MUC1	Mucin 1, transmembrane	(25, 93)	Hs.89603	−1.56	0.0391
PIP5K1B	Phosphatidylinositol-4-phosphate 5-kinase, type I, β	(54)	Hs.297604	−1.56	0.0078
FOXO1A	Forkhead box O1A	(25)	Hs.170133	−1.54	0.023
SAT	Spermidine acetyltransferase 2	(73, 74, 85)	Hs.28491	−1.52	0.0063
BCL2	B-cell CLL/lymphoma 2	(54)	Hs.79241	1.76	0.0009
PCK1	Phosphoenolpyruvate carboxykinase 1	(54)	Hs.1872	1.68	0.0463

Values indicate fold change of each gene in eutopic endometrium from subjects with endometriosis relative to control endometrium.

minichromosome maintenance (MCM) family. Transcripts for five *Mcm* genes were found to be down-regulated, suggesting this pathway to be a major target of progesterone action. Interestingly, our study demonstrated up-regulation of all six MCM genes in the ESE from women with endometriosis. Other genes associated with cell cycle and DNA replication that showed down-regulation in response to progesterone in the study by Pan *et al.* (30) but up-regulation in the ESE from women with endometriosis include PCNA, MKI67, TK1, CCNE1, and MAD2L1. Because progesterone is regarded as the key regulator in shifting the endometrium from the proliferative to the differentiated state (31), our findings suggest that the pathway(s) governing this transition is dysfunctional in the endometrium of subjects with endometriosis.

The molecular mechanisms responsible for the persistence of a proliferative profile in the early secretory endometrium of women with endometriosis are unclear but could result from altered ligand-receptor interactions, coactivators/repressors, or postreceptor signaling (18, 32, 33). We observed differential expression of genes within the progesterone and epidermal growth factor receptor (EGFR) signaling cascades that may be associated with the maintenance of the proliferative fingerprint.

The human PR gene contains several biologically active estrogen response elements (34). Both PR-A and PR-B isoforms are highly expressed in response to estrogen in human endometrium before ovulation, but their expression is down-regulated by progesterone during endometrial maturation (35, 36). In the current study, PR is not suppressed in ESE (fold change 2.12) from women with *vs.* without disease. Previously, an immunohistochemical study reported significantly increased PR expression in the epithelial compartment but not the stromal compartment in the ESE of women with endometriosis (37). In addition, studies have demonstrated differential PR isoform expression in the stromal *vs.* epithelial compartments (38). Because we processed whole endometrium for our gene expression analysis, we cannot comment on compartment-specific PR expression in our endometrial samples.

The FOXO1A gene encodes a progesterone-regulated transcription factor involved in cell cycle control and the induction of apoptosis that is markedly induced on decidualization of endometrial stromal cells in both *in vivo* and *in vitro* assays in response to progesterone and cAMP (39). Our data (ESE fold change −2.27, MSE fold change −1.54) corroborate previous findings by others of reduced FOXO1 gene expression in the endometrium of subjects with endometriosis (40, 41). This finding was confirmed by real-time PCR (ESE fold change −4.00). The reduced FOXO1A expression in the endometrium of subjects with endometriosis relative to controls is consistent with a phenotype of attenuated progesterone response and may play a role in the incomplete

TABLE 6. Differentially expressed genes in the midsecretory phase eutopic endometrium common to both the minimal/mild endometriosis *vs.* normal and moderate/severe endometriosis *vs.* normal data sets

Symbol	Description	Unigene ID	Fold change	*P* value
Up-regulated genes (n = 5)				
S100A8	S100 calcium binding protein A8	Hs.416073	2.11	0.0156
GZMA	Granzyme A	Hs.90708	1.87	0.0286
S100A9	S100 calcium binding protein A9	Hs.112405	1.71	0.0075
C4BPA	Complement component 4 binding protein, α	Hs.1012	1.70	0.0018
KIAA0352	KIAA0352 gene product	Hs.17262	1.55	3.92E-07
Down-regulated genes (n = 12)				
PTPRR	Protein tyrosine phosphatase, receptor type, R	Hs.198288	−2.13	0.0015
SLC15A2	Solute carrier family 15, member 2	Hs.118747	−1.92	0.0107
PLA2G4A	Phospholipase A2, group IVA	Hs.211587	−1.75	0.0397
RBP4	Retinol binding protein 4	Hs.418083	−1.72	$<$0.05
KIAA1199	KIAA1199 protein	Hs.212584	−1.72	0.0349
HLA-DOB	Major histocompatibility complex, class II, DOB	Hs.1802	−1.69	0.0010
ANK3	Ankyrin 3, node of Ranvier (ankyrin G)	Hs.440478	−1.67	0.0202
MUC1	Mucin 1, transmembrane	Hs.89603	−1.56	0.0391
C11orf8	Chromosome 11 open reading frame 8	Hs.432000	−1.56	0.0194
KRT8	Keratin 8	Hs.356123	−1.56	0.0345
PIP5K1B	Phosphatidylinositol-4-phosphate 5-kinase, type I	Hs.297604	−1.56	0.0078
S100A1	S100 calcium binding protein A1	Hs.433503	−1.50	0.0023

Fold change and *P* values are those for the current study.

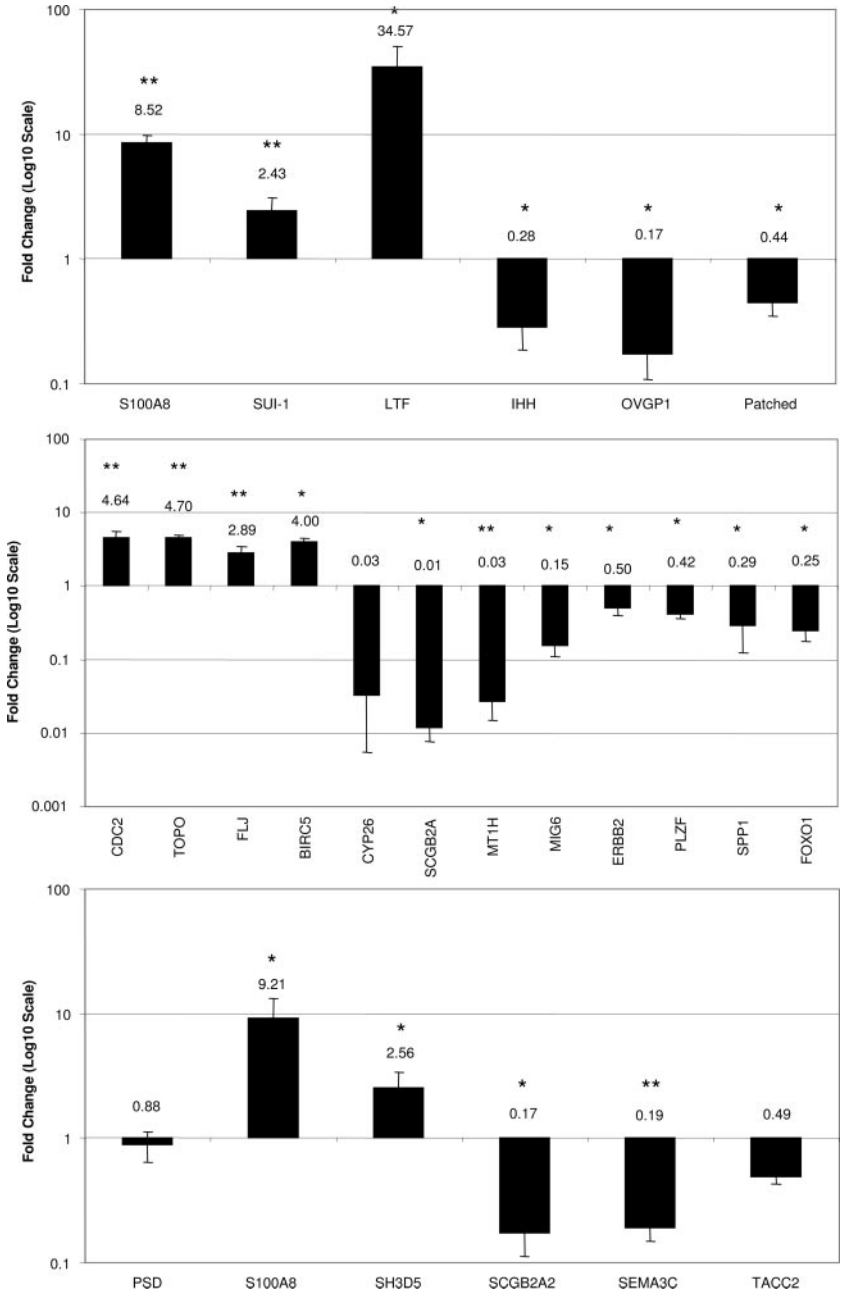

FIG. 3. Expression of selected genes per cycle phase in the endometrium of women with endometriosis relative to women without endometriosis using real-time PCR. *Top*, Proliferative phase. *Middle*, Early secretory phase. *Bottom*, Midsecretory phase. Each phase represents comparison of RNA samples from three women with endometriosis and three women without disease. Fold change values are displayed above each gene and are plotted on the y-axis on a log10 scale. *Bars* represent SEM. *, $P < 0.05$; **, $P < 0.01$.

transitioning of the endometrium from the proliferative-to-early secretory phase.

The molecular mechanism(s) responsible for the persistence of a proliferative profile in the ESE of women with endometriosis may involve nonsteroidal signaling pathways. We observed dysregulation of several antiproliferative genes in the EGFR signaling cascade. Growth factors contribute to maximal proliferation of steroid-dependent cells in normal endometrium (42), and the EGFR pathway is involved in the control of human endometrial growth (43). Mitogen-inducible gene 6 (MIG6) functions as a negative regulator of EGFR-mediated mitogenic signaling. In our data set, MIG6 demonstrated statistically significant down-regulation (fold change −2.70) in ESE of subjects with moderate/severe endometriosis relative to endometrium of subjects without disease and this was validated by real-time PCR (fold change −6.67). Also known as ERFFI1 (for ERBB receptor feedback inhibitor 1), this protein regulates the duration of MAPK activation via attenuation of EGFR autophosphorylation in a mouse knockout model (44). Interestingly, the MIG6 locus (1p36.12–33) falls within a region that is a frequent site of allelic loss in human tumors (45, 46), and a recent study using comparative genomic hybridization to compare the profiles of eutopic and ectopic endometrium in subjects with endometriosis identified shared allelic loss at 1p36 in two of three subjects (47). Down-regulation or loss of MIG6 function may be associated with a conferred survival advantage to the refluxed endometrium in the establishment of endometriotic lesions. Additionally, we observed down-regulation of transducer of ErbB-2

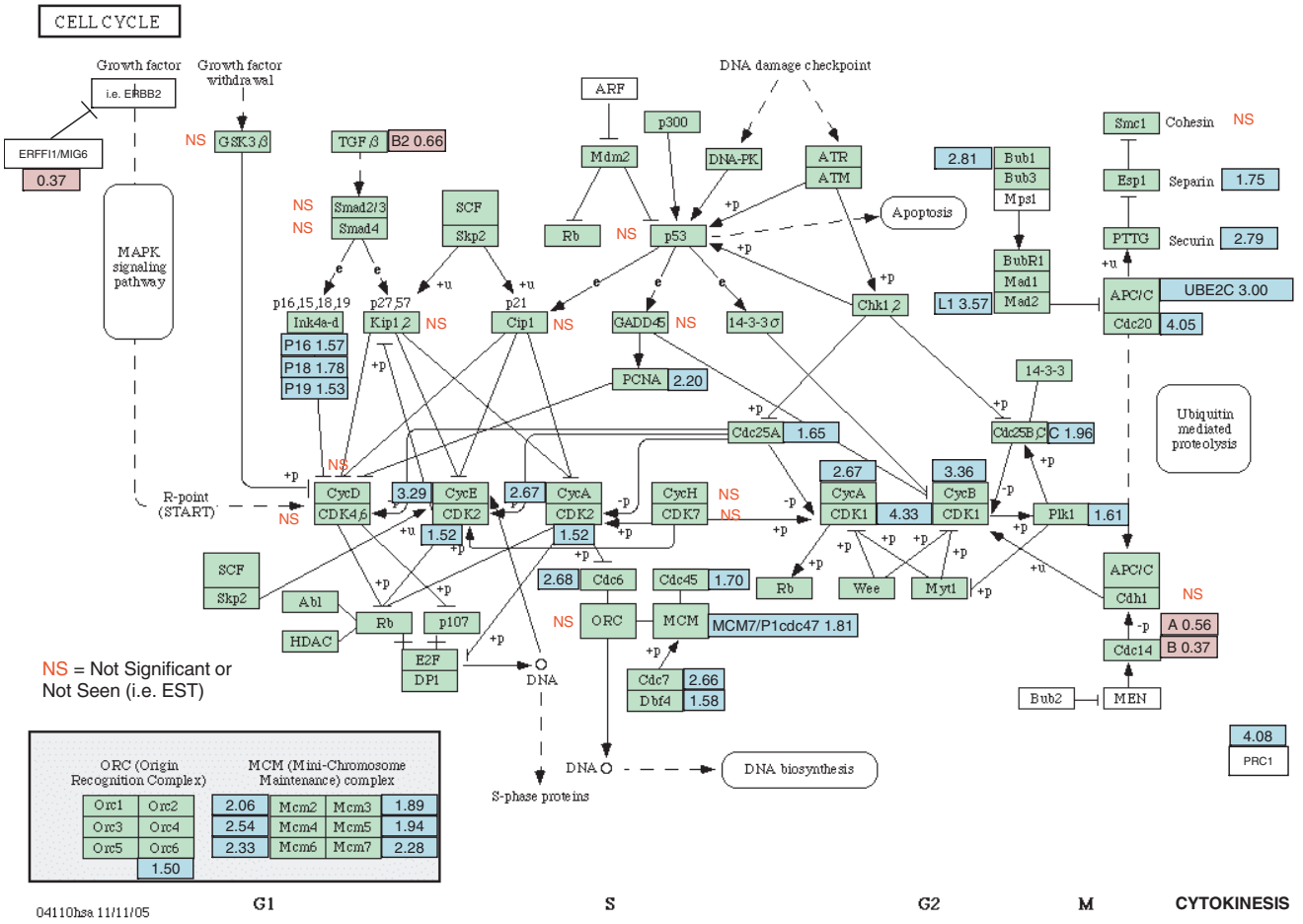

FIG. 4. Differential expression of genes involved in the regulation of the mitotic cell cycle in ESE from women with *vs.* without endometriosis. In this diagram, each *box* represents a particular gene. Up-regulated genes with fold change are represented in *green*, whereas down-regulated genes and fold change are represented in *red*. Diagram adapted from KEGG (http://www.genome.jp.kegg).

(TOB1; fold change −2.44) in the ESE of women with endometriosis. TOB1 is a cell cycle-regulatory protein associated with antiproliferative activity (48). Studies of cultured human endometrial stromal cells from women with endometriosis demonstrated reduced TOB1 expression after treatment with IL-1β, a central cytokine in endometriosis (49). The TOB1 gene is located on chromosome 17q21, and functional loss of this chromosomal region has been observed in endometriotic lesions (50). The differential expression of several genes involved in checking the mitogenic action of the EGFR signaling cascade is intriguing. Further study is necessary to explore the molecular cross talk between progesterone and the EGFR signaling cascade in the control of endometrial growth and decidualization in women with and without endometriosis.

Dysregulation of progesterone target genes in the secretory endometrium of women with endometriosis

In addition to genes involved in cellular proliferation, the secretory phase profiles of many progesterone-regulated genes in eutopic endometrium of women with endometriosis provide further evidence of a relative reduction in progesterone response. Fifty-four genes in the ESE and 16 genes in the MSE evidenced dysregulation in women with disease (Table 5). Metallothioneins (MTs) comprise a family of genes clustered on chromosome 16q that bind to heavy metal ions and minimize reactive oxygen species. Previous studies demonstrated high MT expression in the secretory phase endometrium of women without endometriosis (25) and low MT expression in endometriotic implants (51). In the present study, the MTs were among the most highly down-regulated genes in the ESE of women with endometriosis, and this was validated by real-time PCR (MT1H fold change −33.33). Glutathione peroxidase, also up-regulated during the secretory phase in normal endometrium, shares the MT pathway and evidenced significantly reduced expression (ESE fold change −2.78) in the eutopic endometrium of women with endometriosis. The antiapoptotic gene, BCL-2, is increased in ESE of women with endometriosis, confirming studies by others (52, 53) and suggesting mechanisms for enhanced cell survival in the pathogenesis of this disorder. Interestingly, this gene is negatively regulated by progesterone in mouse uterus (54). Another progesterone-regulated gene evidencing striking dysregulation in the endometrium of subjects with endometriosis is CYP26A1. In normal premenopausal

endometrium, the gene expression of this retinoic acid catabolic enzyme markedly increases in the secretory phase (55). In a microarray study comparing genes induced by progesterone in the uteri of wild-type *vs.* PR knockout mice, CYP26A1 was the most highly up-regulated gene in response to progesterone (54). In women with moderate/severe endometriosis relative to controls, CYP26A1 is among the most significantly down-regulated genes in both the ESE and MSE, with fold changes of −8.33 and −2.63, respectively, and validated by real time RT-PCR. Interestingly, the genetic locus for CYP26A1 maps close to a region of the genome recently identified to be significantly associated with endometriosis in a genome-wide linkage study (29).

Clinical implications of attenuated progesterone action: implantation failure

An association between endometriosis and infertility is well established (56–61). Attenuation of progesterone response at the level of the endometrium may be expected to have a deleterious impact on endometrial receptivity, and a significant reduction of the implantation rate in women with endometriosis undergoing *in vitro* fertilization has been reported (62). Our prior study identified an altered transcriptome in the endometrium of women with minimum/mild endometriosis during the window of implantation (8). Systematic comparison of the list of differentially expressed genes in the midsecretory phase of the current study with that of the prior study showed 17 genes to be common (Table 6). In the context of attenuated progesterone response and implantation failure, several genes are of interest. MUC-1 and osteopontin, important in embryo attachment, and glycodelin, important in the immune response during implantation, were down-regulated in secretory endometrium of women with *vs.* without endometriosis. We observed a nearly 2-fold reduction in expression of IGF binding protein (IGFBP)-1 during the window of implantation in the endometrium from women with disease. IGFBP-1 is a sensitive marker for endometrial stromal cell decidualization, and a reduction in IGFBP-1 secretion by cultured endometrial stromal fibroblasts from women with endometriosis relative to those from women without disease has been documented (63). These findings suggest impaired decidualization of the endometrium in women with endometriosis, which may have important biochemical implications for uterine receptivity.

Mechanism of attenuated progesterone response

Herein we have demonstrated abnormalities in eutopic endometrium of women with endometriosis, primarily in the early secretory phase, suggestive of reduced progesterone response in the transition from the proliferative to secretory phases. In addition, a number of progesterone-regulated genes evidence dysregulation in secretory phase endometrium. Whether these changes in the endometrial transcriptome are secondary to reduced progesterone responsiveness at the level of the endometrium or to a lower level of circulating or local bioavailable progesterone is unclear. However, *in vivo* observations and *in vitro* studies suggest an intrinsic resistance to progesterone action in eutopic endometrium of women with endometriosis.

Progesterone resistance exists when normal levels of progesterone elicit a subnormal or reduced response. Studies are conflicting regarding the normalcy of circulating levels of progesterone in women with endometriosis (64–68), and this discrepancy may be secondary to difficulties in both ascertainment and interpretation of circulating progesterone levels. A single serum progesterone level may not be representative of luteal adequacy (69, 70), and successful intrauterine pregnancy has been documented with midluteal progesterone levels as low as 3–4 ng/ml (70, 71). Finally, a study of luteal endometrial differentiation in programmed cycles of physiological and subphysiological exogenous progesterone replacement in GnRH agonist-suppressed healthy volunteers showed no differences in endometrial thickness, histology, or epithelial integrin expression at the lower serum progesterone level (72). This finding supports the argument that the reduced progesterone response in the eutopic endometrium of women with endometriosis is an intrinsic biologic alteration of the endometrium.

The evidence to support progesterone resistance in the setting of endometriosis is substantial. Endometrial stromal fibroblasts obtained from eutopic endometrium and ectopic endometrium (endometriotic lesions) demonstrate impaired ability to decidualize *in vitro*, a finding highly suggestive of an intrinsic abnormality in the progesterone-signaling pathway (63). Others have observed dysregulation of progesterone target genes in cultured endometrial stromal cells from women with endometriosis, significant insofar as the progesterone level in the culture medium is well controlled (15). A model for progesterone resistance based on differential PR isoform expression has been described for ectopic endometrium (20), and a reduced responsiveness to progesterone in eutopic endometrium has been implicated in disease pathogenesis (14). Our gene expression findings are consistent with resistance to progesterone action in the endometrium of women with endometriosis. The current study provides a framework for further investigation as to the mechanism(s) underlying attenuated progesterone response in eutopic endometrium of women with endometriosis.

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