


Molecular Evidence for Differences in Endometrium in Severe Versus Mild Endometriosis

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Lusine Aghajanova, MD, PhD¹, and
Linda C. Giudice, MD, PhD, MSc¹

Abstract

Women with stage III/IV versus stage I/II endometriosis have lower implantation and pregnancy rates in natural and assisted reproduction cycles. To elucidate potential molecular mechanisms underlying these clinical observations, herein we investigated the transcriptome of eutopic endometrium across the menstrual cycle in the setting of severe versus mild endometriosis. Proliferative (PE), early secretory (ESE), and mid-secretory (MSE) endometrial tissues were obtained from 63 participants with endometriosis (19 mild and 44 severe). Purified RNA was subjected to microarray analysis using the Gene 1.0 ST Affymetrix platform. Data were analyzed with GeneSpring and Ingenuity Pathway Analysis and subsequently validated. Comparison of differentially regulated genes, analyzed by cycle phase, revealed dysregulation of progesterone and/or cyclic adenosine monophosphate (cAMP)-regulated genes and genes related to thyroid hormone action and metabolism. Also, members of the epidermal growth factor receptor (EGFR) signaling pathway were observed, with the greatest upregulation of EGFR in severe versus mild disease during the early secretory phase. The extracellular matrix proteoglycan versican (VCAN), which regulates cell proliferation and apoptosis, was the most highly expressed gene in severe versus mild disease. Upregulation of microRNA 21 (MIR21) and DICER1 transcripts suggests roles for microRNAs (miRNAs) in the pathogenesis of severe versus mild endometriosis, potentially through regulation of gene silencing and epigenetic mechanisms. These observed differences in transcriptomic signatures and signaling pathways may result in poorly programmed endometrium during the cycle, contributing to lower implantation and pregnancy rates in women with severe versus mild endometriosis.

Keywords

severe endometriosis, mild endometriosis, eutopic endometrium, microarray, transcriptome

Introduction

Endometriosis is a benign gynecologic disease characterized by endometrial-like tissue (epithelium and stroma) outside the uterus. It affects primarily women of reproductive age and presents with pelvic pain and infertility.^{1,2} Endometriosis is diagnosed mainly by visualization at surgery, and the revised American Society for Reproductive Medicine (ASRM) staging system recognizes minimal, mild, moderate, and severe (I-IV) stages of disease, based on the number and character of peritoneal lesions, ovarian and other organ involvement, and presence, type, and extent of adhesions.^{3,4} Although peritoneal, ovarian and rectovaginal endometriotic lesions are considered distinct entities with different pathogenesis,⁵⁻⁷ mild and severe stages of peritoneal endometriosis may also be distinct disorders, though the supporting data are limited. However, there is clinical evidence that embryonic implantation rates differ in women with severe versus mild endometriosis (see below), suggesting that the eutopic endometrium is different functionally and biochemically in these 2 types of endometriosis.

Women with moderate–severe endometriosis have more difficulty conceiving, compared to those with minimal–mild disease.⁸ Also, women with stage III/IV endometriosis have significantly lower implantation rates (13.7% vs 28.3%, respectively; $P < .05$) and pregnancy rates (22.6% vs 40.0%, respectively; $P < .01$) but not fertilization or miscarriage rates, compared to women with stage I/II endometriosis.⁹ A meta-analysis of 22 published studies on endometriosis and in vitro fertilization (IVF) outcomes showed that IVF pregnancy rates are significantly lower in women with severe versus mild endometriosis (13.84% vs 21.12%,

¹Department of Obstetrics, Gynecology and Reproductive Sciences, University of California–San Francisco (UCSF), San Francisco, CA, USA

Corresponding Author:

Linda C. Giudice, Department of Obstetrics, Gynecology and Reproductive Sciences, The Robert B. Jaffe, MD Endowed Professor in the Reproductive Sciences, University of California, San Francisco, 505 Parnassus Ave., M1496, Box 0132, San Francisco, CA 94143, USA
Email: giudice@obgyn.ucsf.edu

respectively; $P < .001$),¹⁰ underscoring a potential endometrial origin of these differences. Also, participants with advanced disease demonstrate diminished ovarian response and higher cancellation rates in IVF cycles, but improved implantation, pregnancy, miscarriage, and delivery rates, after surgery, similar to those for women with tubal factor infertility,¹¹ suggesting that removal of disease improves endometrial receptivity.

We have previously compared the transcriptome of eutopic endometrium from women with minimal/mild disease with the endometrium from women without disease during the window of implantation (mid-secretory endometrium [MSE])¹² and also the endometrial transcriptome from women with moderate/severe disease compared with no disease in proliferative (PE), early secretory endometrium (ESE), and MSE.¹³ Based on these and other studies,¹⁴ endometrium from women with endometriosis appears to differ from that of disease-free women.^{12,13,15} Herein, we compared the transcriptome of eutopic endometrium from women with severe versus mild endometriosis at different times in the menstrual cycle, in an attempt to understand the differences and their potential roles contributing to the pathophysiology of infertility in women with endometriosis.

Materials and Methods

Study Participants

The study was approved by the Committee on Human Research of the University of California—San Francisco (UCSF) and the Stanford University Committee on the Use of Human Subjects in Medical Research. Samples were obtained from the National Institute of Health Specialized Cooperative Centers Program in Reproduction and Infertility Research (NIH SCCPRR) Human Endometrial Tissue and DNA Bank at UCSF. Endometrial tissue was obtained from 12 participants without endometriosis undergoing endometrial biopsy or hysterectomy for benign disorders not related to endometrial pathology (used in immunohistochemistry experiments) and from 63 participants with endometriosis undergoing endometrial biopsy for infertility evaluation or hysterectomy for treatment of severe pelvic pain and extensive endometriosis (Table 1). All participants were documented not to be pregnant and not to have had hormonal treatment for at least 3 months before surgery. Staging of endometriosis was performed according to the revised American Fertility Society classification system.^{3,4} Of the 63 participants, 19 had mild and 44 had severe endometriosis. The majority of endometriosis samples were obtained by endometrial biopsy, whereas the majority of control (no endometriosis) samples were obtained after hysterectomy (Table 1). (Note our previous studies demonstrated that sampling technique does not affect the endometrial transcriptome.¹⁶) The mean ages (years) of patients were: mild endometriosis group, 35.7 ± 1.4 ; severe endometriosis group, 35.2 ± 1.21 ($P > .05$). Menstrual cycle phase was determined based on histological evaluation of the tissue by 3 independent readers and according to the Noyes' criteria.¹⁷

Isolation of RNA and Preparation for Hybridization

Each endometrial tissue specimen was processed individually for microarray hybridization, as described earlier.¹³ Briefly, total RNA was extracted from whole-tissue specimens using the Trizol reagent (Invitrogen, Carlsbad, California), subjected to DNase treatment, and purified using the RNeasy Plus Kit (QIAGEN, Valencia, California). RNA purity was assessed by the A260/A280 ratio, and quality and integrity were assessed using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, California), with all samples having high-quality RNA (RNA Integrity Number (RIN) = 9.7–10).

RNA samples were prepared for microarray analysis according to the Affymetrix protocol (Affymetrix, Inc, Santa Clara, California), as described earlier.^{13,16} Briefly, for each sample, 5 μg of total RNA were reverse transcribed to complementary DNA (cDNA). Second strand DNA was generated using DNA polymerase, followed by overnight *in vitro* transcription to generate cRNA. After chemical fragmentation, biotinylated cRNAs were ready for hybridization. Quality of the final product was assessed in the Agilent Bioanalyzer. Each sample was hybridized to HU133 Plus 2.0 high-density oligonucleotide array (Affymetrix), with 54 600 genes and expressed sequence tags (ESTs), at the UCSF Genomic Core Facility. The data were scanned according to the protocol described in Assay Manual from Affymetrix.

Microarray Data Analysis

The .cel data files were imported into GeneSpring GX 10.0 software (Agilent Technologies) and processed using the robust multiarray analysis (RMA) algorithm for background adjustment, normalization, and log₂-transformation of perfect match (PM) values.¹⁶ The data during each menstrual cycle phase (PE, ESE, and MSE) were compared between the severe and mild endometriosis groups. The generated gene lists included only genes with >2.0-fold change (FC) and $P < .05$ by 1-way analysis of variance (ANOVA) with Tukey post hoc test and Benjamini-Hochberg multiple testing correction for false discovery rate.

Principal Component Analysis and Hierarchical Clustering

Principal component analysis (PCA) and hierarchical clustering were performed as described.^{15,16} Principal component analysis is an unbiased analysis performed in GeneSpring with all samples, using all 42 203 genes and 12 397 ESTs on Affymetrix Human HU133 Plus 2.0 arrays to look for similar expression patterns and underlying cluster structures. Hierarchical cluster analysis of differentially expressed genes from all samples was conducted using the smooth correlation distance measure algorithm (GeneSpring) to identify samples with similar patterns of gene expression. Compared to PCA, hierarchical clustering uses only informational genes—that is are differentially expressed among all experimental conditions.

Table 1. Characteristics of Participants and Endometrial Tissue Samples in the Study

Participant ID	Cycle Phase	Experiment	How Endometrium was Obtained	Age	Ethnicity
Whole tissue biopsy used for microarray analysis and validations					
Mild endometriosis					
609	PE	Microarray, IHC	Endometrial biopsy	27	Mixed
621	PE	Microarray, IHC	Hysterectomy	37	Caucasian
658	PE	Microarray, QPCR, IHC	Endometrial biopsy	36	Caucasian
660	PE	Microarray, QPCR	Endometrial biopsy	46	Caucasian
ST-007	PE	Microarray, QPCR	Endometrial biopsy	41	Unknown
ST-012	PE	Microarray, QPCR,	Endometrial biopsy	41	Unknown
ST-042	PE	Microarray	Endometrial biopsy	34	Caucasian
ST-071	PE	Microarray	Endometrial biopsy	42	Caucasian
ST-082	PE	Microarray, IHC	Endometrial biopsy	32	Caucasian
ST-50	PE	Microarray, QPCR	Endometrial biopsy	39	Caucasian
ST-080	ESE	Microarray, QPCR, IHC	Endometrial biopsy	43	Unknown
ST-089	ESE	Microarray, QPCR, IHC	Endometrial biopsy	33	Caucasian
ST-113	ESE	Microarray, QPCR, IHC	Endometrial biopsy	27	Caucasian
550	MSE	Microarray	Endometrial biopsy	38	Mixed
ST-009	MSE	Microarray, QPCR, IHC	Endometrial biopsy	31	Caucasian
ST-014	MSE	Microarray, QPCR, IHC	Endometrial biopsy	28	Black
ST-033	MSE	Microarray, QPCR	Endometrial biopsy	42	Caucasian
ST-038	MSE	Microarray, QPCR	Endometrial biopsy	36	Caucasian
ST-121	MSE	Microarray, QPCR, IHC	Endometrial biopsy	25	Caucasian
Severe endometriosis					
26A ^a	PE	Microarray	Endometrial biopsy	31	Caucasian
508 ^a	PE	Microarray, QPCR	Endometrial biopsy	25	Caucasian
511	PE	Microarray	Endometrial biopsy	42	Caucasian
575 ^a	PE	Microarray, IHC	Endometrial biopsy	26	Unknown
587 ^a	PE	Microarray, QPCR	Endometrial biopsy	37	Caucasian
589	PE	Microarray, IHC	Hysterectomy	48	Asian
594 ^a	PE	Microarray	Endometrial biopsy	38	Caucasian
595	PE	Microarray	Endometrial biopsy	37	Asian
647 ^a	PE	Microarray, QPCR	Endometrial biopsy	39	Caucasian
651 ^a	PE	Microarray, QPCR	Endometrial biopsy	37	Caucasian
ST-049	PE	Microarray	Endometrial biopsy	29	Caucasian
ST-076	PE	Microarray	Endometrial biopsy	30	Hispanic
ST-084	PE	Microarray, QPCR, IHC	Endometrial biopsy	37	Caucasian
ST-090	PE	Microarray, QPCR, IHC	Endometrial biopsy	42	Caucasian
ST-70	PE	Microarray	Endometrial biopsy	22	Caucasian
489 ^a	ESE	Microarray, IHC	Hysterectomy	39	Asian
496 ^a	ESE	Microarray, QPCR, IHC	Endometrial biopsy	37	Caucasian
517 ^a	ESE	Microarray	Endometrial biopsy	35	Asian
599 ^a	ESE	Microarray, IHC	Endometrial biopsy	35	Black
607	ESE	Microarray, IHC	Endometrial biopsy	24	Asian
684	ESE	Microarray, QPCR, IHC	Hysterectomy	36	Caucasian
27A ^a	ESE	Microarray	Endometrial biopsy	22	Caucasian
ST-036	ESE	Microarray	Endometrial biopsy	45	Caucasian
ST-065	ESE	Microarray, QPCR	Endometrial biopsy	34	Asian
ST-112	ESE	Microarray, QPCR, IHC	Endometrial biopsy	38	Caucasian
ST-127	ESE	Microarray, IHC	Endometrial biopsy	43	Caucasian
ST-130	ESE	Microarray, QPCR, IHC	Endometrial biopsy	35	Caucasian
516 ^a	MSE	Microarray	Endometrial biopsy	34	Asian
526	MSE	Microarray, QPCR, IHC	Hysterectomy	48	Unknown
540 ^a	MSE	Microarray	Endometrial biopsy	37	Caucasian
543 ^a	MSE	Microarray	Endometrial biopsy	38	Caucasian
544	MSE	Microarray, QPCR, IHC	Endometrial biopsy	46	Caucasian
645 ^a	MSE	Microarray	Endometrial biopsy	39	Asian Indian
678 ^a	MSE	Microarray	Hysterectomy	44	Asian
33A ^a	MSE	Microarray	Endometrial biopsy	27	Caucasian
72A ^a	MSE	Microarray	Endometrial biopsy	31	Caucasian

(continued)

Table 1 (continued)

Participant ID	Cycle Phase	Experiment	How Endometrium was Obtained	Age	Ethnicity
73A ^a	MSE	Microarray	Endometrial biopsy	26	Caucasian
97A ^a	MSE	Microarray	Endometrial biopsy	35	Unknown
ST-037	MSE	Microarray, QPCR, IHC	Hysterectomy	32	Caucasian
ST-039	MSE	Microarray, QPCR	Endometrial biopsy	44	Caucasian
ST-078	MSE	Microarray	Endometrial biopsy	32	Caucasian
ST-091	MSE	Microarray, QPCR	Endometrial biopsy	20	Caucasian
ST-096	MSE	Microarray, QPCR, IHC	Endometrial biopsy	31	Caucasian
ST-119	MSE	Microarray, IHC	Endometrial biopsy	41	Asian
No endometriosis					
455 ^b	PE	IHC	Hysterectomy	39	Caucasian
469	PE	IHC	Hysterectomy	42	Caucasian
604	PE	IHC	Hysterectomy	44	Caucasian
693	PE	IHC	Hysterectomy	46	Caucasian
UC-24	ESE	IHC	Hysterectomy	45	Black
UC-26	ESE	IHC	Hysterectomy	34	Caucasian
629	ESE	IHC	Hysterectomy	46	Caucasian
680	ESE	IHC	Hysterectomy	34	Caucasian
463	MSE	IHC	Hysterectomy	48	Caucasian
501	MSE	IHC	Hysterectomy	49	Caucasian
610 ^b	MSE	IHC	Hysterectomy	50	Caucasian
626 ^b	MSE	IHC	Hysterectomy	42	Caucasian

Abbreviations: QPCR, quantitative real-time reverse transcriptase–polymerase chain reaction; IHC, immunohistochemistry; PE, proliferative endometrium; ESE, early secretory endometrium; MSE, mid-secretory endometrium.

^a Samples used in Burney et al, 2007.¹³

^b Samples used in Talbi et al, 2006.¹⁶

Ingenuity Pathway Analysis

Gene symbols and FCs of the up- and downregulated genes in each pairwise comparison were imported into Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, California), as described earlier.¹⁵ For each comparison, associated top significantly regulated molecular and biological networks and canonical molecular pathways were identified. Only networks with the highest score were selected for the analysis. This was followed by functional analysis on the data set level and canonical pathway analysis. The significance of the association between the genes from the data set and the canonical pathway (in the IPA library) was presented as a ratio of the number of genes from the data set in a given pathway divided by the total number of molecules that make up the canonical pathway (Fisher exact test was used to calculate a *P* value). Pathways with *P* < .05 and ratio >0.05 were considered significant.

Microarray Validation by Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was performed in duplicate using the SYBR Green PCR Mix (Fermentas Inc, Glen Burnie, Maryland), according to the manufacturer's instructions. The housekeeping gene *RPL19* was used as the normalizer. Numbers of mild endometriosis samples used for validation were *n* = 5, *n* = 3, and *n* = 5 for PE, ESE, and MSE, respectively, and in the severe endometriosis group, *n* = 6, *n* = 5, and *n* = 6 for PE, ESE, and MSE, respectively (Table 1). The following primer sequences were

used: thyroxine deiodinase 2 (*DIO2*) sense 5'- TTGTAC TTACTCTAAATTTCCCAAGG-3' and antisense 5'-CATTG CCACTGTTGT CACCT-3'; insulin-like growth factor binding protein 5 (*IGFBP5*) sense 5'-TGCACCTGAGATGAGACAGG-3' and antisense 5'-GCTTCATCCCCTACTTGTCC-3'; somatostatin (*SST*) sense 5'-CCCAGACTCCGTCAGTTTCT-3' and antisense 5'-ATCATTCTCCGCTGTTGG-3'; transgelin (*TAGLN*) sense 5'-TTAGCTTTCCCCAGACATGG-3' and antisense 5'-CGGTA GTGCCATCATTCTT-3'; versican (*VCAN*) sense 5'-CCAGC CCCCTGTTGTAGAAA-3' and antisense 5'-ATTGAATTGTC CTTT GCTGATG-3'; solute carrier family 1, member 1 (*SLC1A1*) sense 5'-AACACTGCCTGTCACCTTCC-3' and antisense 5'-GC ACTCAGCACAATCACCAT-3'; epidermal growth factor receptor (*EGFR*) sense 5'-GAATGCATTTGCCAAGTCT-3' and antisense 5'-CGTCTATGCTGTCTCAGTCA-3'; and *RPL19* sense 5'-GCA GAT AATGGGAGGAGCC-3' and antisense 5'- GC CCATCTTT GATG AGCTTC-3'. Polymerase chain reactions were run on the Mx4000 and Mx3005 quantitative real-time reverse transcriptase–polymerase chain reaction (QPCR) Stratagene systems (Agilent Technologies), using thermal cycling conditions, as described.^{15,18} Statistical analysis for the QRT-PCR results was performed using the nonparametric Mann-Whitney test. Significance was determined at *P* ≤ .05.

Immunohistochemistry

Immunostaining was performed for *VCAN* and *EGFR* using 4 μm thick paraffin-embedded endometrial tissue sections from women with mild and severe endometriosis: PE, *n* = 4

and $n = 5$, respectively; ESE, $n = 3$ and $n = 7$, respectively; and MSE, $n = 3$ and $n = 5$, respectively), as well as women without endometriosis ($n = 4$ in all phases). The samples were de-paraffinized in Xylene (Sigma-Aldrich, St Louis, Missouri) and rehydrated in decreasing concentrations of ethanol. All slides were incubated for 15 minutes in H_2O_2 (3% in methanol) to block endogenous peroxidase activity after antigen retrieval by boiling slides in citrate buffer (pH = 6.0). Thereafter the slides were blocked with normal horse serum for 45 minutes, followed by incubation with the primary antibody: overnight $4^\circ C$ incubation with the rabbit polyclonal anti-VCAN antibody (Versican V0/V2 Neo, ThermoScientific, Waltham, Massachusetts) at $5 \mu g/mL$ concentration, and 1 hour at room temperature for the rabbit anti-human EGFR antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, California, kind gift from Dr M Hsieh, UCSF) at 1:50 dilution.

In negative control slides, the primary antibody was replaced with nonimmune immunoglobulin G (IgG) of equivalent concentration from the same species. All slides were incubated with universal goat anti-rabbit/mouse secondary antibodies (Vector Laboratories Inc, Burlingame, California) for 30 minutes at room temperature. A freshly prepared diaminobenzidine-hydrogen peroxide solution (ImmPACT DAB kit, Vector Laboratories) was added to the slides, which were thereafter rinsed with distilled water. The slides were counterstained with haematoxylin (Vector Laboratories) and mounted with Clarion mounting medium (SigmaAldrich). A Leica microscope was used to visualize the immunostaining and to photograph the results. Sections of mouse ovarian and lung tissue (a kind gift from Dr Marco Conti, UCSF) were used as positive controls for VCAN immunostaining.^{19,20} Sections of 12-week human placental tissue served as a positive control for EGFR staining²¹; myometrium served as an internal positive control.^{22,23}

Results

Cluster Analysis

Principal component analysis of all genes showed that mild and severe endometriosis samples cluster according to their cycle phase rather than the disease stage (Figure 1A), confirming previous observations of phase-dependent segregation when analyzing endometrial tissue or isolated cells.^{13,15} However, PCA followed by subsequent analysis of disease stage demonstrated that severe endometriosis samples cluster separately from mild endometriosis, regardless of cycle phase, although there was some overlap (Figure 1B).

Unsupervised hierarchical clustering analysis was conducted using the profiles of significantly regulated genes in each study group (Figure 1C, clusterogram). Severe endometriosis samples clustered together and separately from mild endometriosis samples. Early secretory endometriosis from the mild endometriosis group clustered close to the mild PE group. Remarkably, even though clustering analysis of mild and severe endometriosis samples showed that they

clustered separately from each other, signifying the difference between these 2 stages of endometriosis, PE as well as ESE and MSE samples from all groups demonstrated branching from the same stem, supporting the conclusion that cycle phase has greater impact than disease stage in sample clustering (Figure 1C).

Endometrial Transcriptome

Severe versus mild endometriosis. Comparison of severe versus mild endometriosis samples in the proliferative phase revealed 380 differentially regulated genes ($P < .05$, FC = 2; Supplement Table 1), with 120 up- and 260 downregulated. Transcripts for several extracellular matrix (ECM) proteins and their receptors, such as VCAN, laminin- $\beta 1$, fibrillin 1, and integrin- $\beta 1$ (fibronectin receptor), were upregulated in severe endometriosis PE samples, as were heat shock proteins, DIO2 (the enzyme that converts thyroxine T_4 to triiodothyronine T_3), thioredoxin interacting protein (TXNIP), relaxin/insulin-like family peptide receptor 1, EGFR, microRNA 21 (MIR21), interferon- γ receptor 1, neuropilin, and others (Supplement Table 1).

Comparison of severe versus mild endometriosis samples in the early secretory phase revealed 817 differentially regulated genes (166 up- and 651 downregulated; Supplement Table 2). Although dysregulation of some genes persisted from the proliferative phase, some new genes were revealed, including upregulation of CYP26A1, IGF1, DICER1, DUSP1, KLF9, PAPPA, FOXO1A, neurotrophic tyrosine kinase receptor type 3, transducer of ERBB2 (TOB), and sulfatase 2 and downregulation of thyrotropin-releasing hormone (TRH), SST, lactotransferrin (LTF), TAGLN, Indian hedgehog homolog (IHH), BMP7, CXCL14, and others (Supplement Table 2). Some of the upregulated genes are progesterone and/or estradiol dependent, although some known progesterone-regulated genes (eg, *IGFBP6*, secretoglobulin family 3A1, complement D, and glutathione peroxidase 3 [*GPX3*]) were downregulated. These data suggest that the steroid hormone response and intracellular programs are disordered in both severe and mild forms of endometriosis in the early secretory phase.

Comparison of severe versus mild endometriosis samples in the mid-secretory phase revealed 1286 differentially regulated genes (Supplement Table 3), with 377 and 909 genes being up- and downregulated, respectively. These data are consistent with the hierarchical clusterogram and indicate that the greatest differences between severe and mild endometriosis occur in the window of implantation (Figure 1C). Interestingly, some progesterone-regulated genes such as *DKK1*, *MAOA*, *MAOB*, *CXCL14*, *IL15*, *IL1R1*, *IDO1*, and *CD55* were upregulated in this comparison group, although other progesterone-regulated genes, for example, *KLF-13*, *IGFBP6*, and members of the Notch-signaling pathway were downregulated (Supplemental Table 3). These data are consistent with dysregulation in the response to progesterone in MSE in both forms of endometriosis, as observed in ESE.

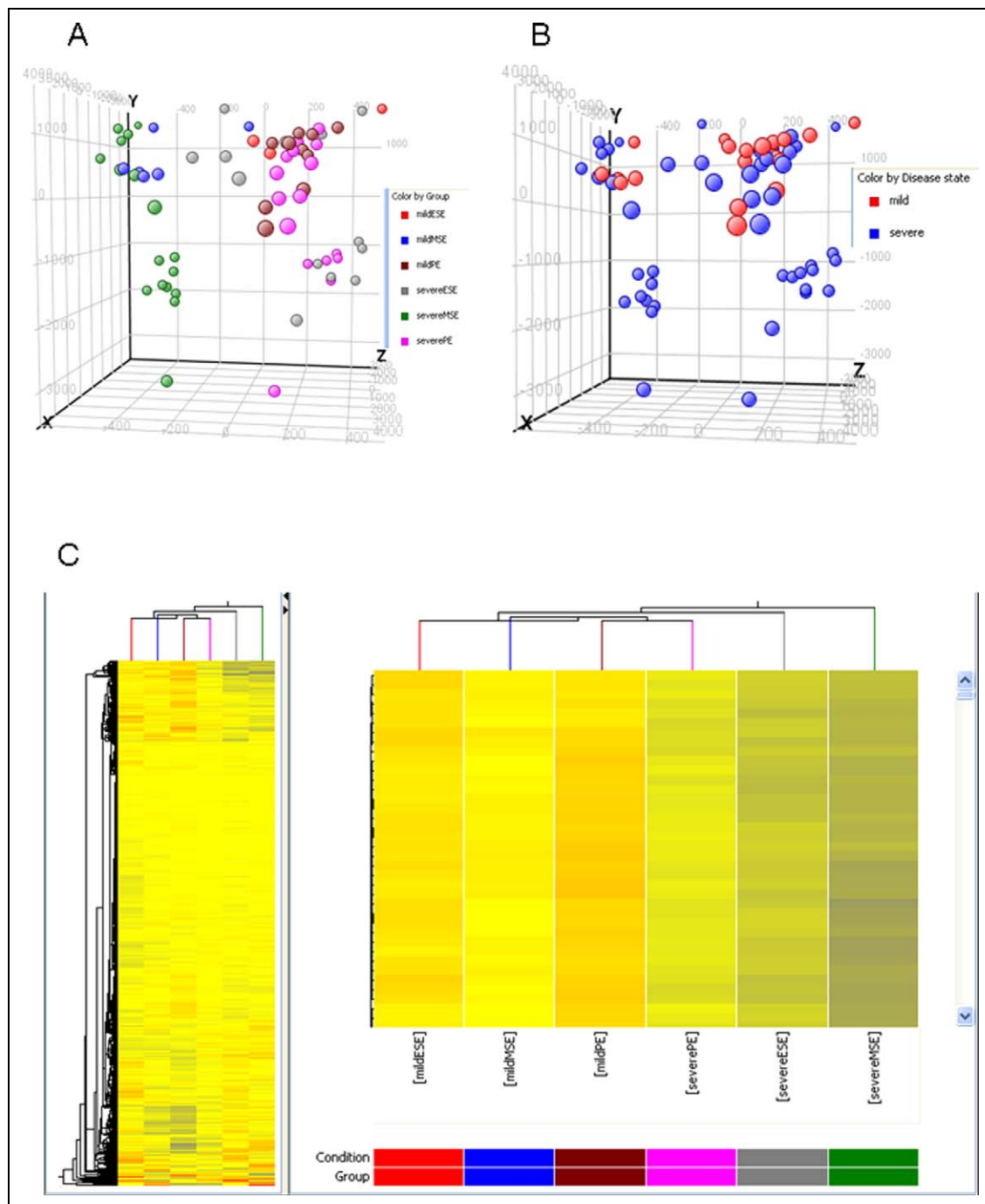


Figure 1. Clustering analyses of samples from participants with mild and severe endometriosis. Panels A and B, Principal component analysis (PCA) of samples. A, Analyzed by menstrual cycle phase; and B, Analyzed by disease stage. PCA was applied to all endometrial samples that were characterized by the gene expression of all probes on the Affymetrix Gene 1.0 ST platform. C, Hierarchical clustering analysis of no-endometriosis and mild and severe endometriosis samples throughout the menstrual cycle, using the profiles of significantly regulated genes. PE indicates proliferative phase endometriosis; ESE, early secretory phase endometriosis; MSE, mid-secretory phase endometriosis; m, mild endometriosis; s, severe endometriosis.

Gene Ontology Categories in Severe Versus Mild Endometriosis Throughout the Menstrual Cycle

The most common gene ontology (GO) biological process groups in all comparisons were transcription, transport, cell adhesion,

nuclear messenger RNA (mRNA) splicing, proteolysis, translation, and cell cycle, with angiogenesis and apoptosis processes having significant representation in the secretory (ESE and MSE) phase (Table 2). The main cellular components involved were nucleus, cytoplasm, extracellular and intracellular regions, and

Table 2. The Most Represented Gene Ontology (GO) Categories in Severe Endometriosis

GO Biological Process	GO Cellular Component	GO Molecular Function
PE vs mild endometriosis PE		
Transcription	Nucleus	Nucleotide binding
Transport	Intracellular	Protein binding
Cell adhesion	Cytoplasm	DNA binding
Nuclear mRNA splicing, via spliceosome	Extracellular region	Receptor activity
Proteolysis	Membrane	Nucleic acid binding
Translation	Mitochondrion	Catalytic activity
Signal transduction	Plasma membrane	Actin binding
Negative regulation of transcription from RNA polymerase II promoter	Membrane fraction	Binding
Lipid metabolic process	Integral to plasma membrane	Structural constituent of ribosome
Protein folding	Golgi membrane	Signal transducer activity
Regulation of transcription, DNA-dependent	Endoplasmic reticulum	Structural molecule activity
Skeletal system development	Integral to membrane	Zinc ion binding
Mesoderm formation	Golgi apparatus	Magnesium ion binding
Cell cycle	Ruffle	Receptor binding
Protein amino acid phosphorylation	Cytosol	Insulin receptor binding
Ubiquitin-dependent protein catabolic process	Cytoskeleton	RNA binding
Carbohydrate metabolic process	Extracellular space	Transporter activity
Mitochondrial electron transport, NADH to ubiquinone	Filopodium	NADH dehydrogenase activity
Multicellular organismal development	Mitochondrial inner membrane	Hydrolase activity
RNA processing	Eukaryotic translation initiation factor 4F complex	Ion channel activity
mRNA processing	Nucleolus	Calcium ion binding
Organ morphogenesis	Cornified envelope	Metalloendopeptidase activity
Cell fate determination	Endosome	Transcription factor activity
Angiogenesis	Inner acrosomal membrane	Ubiquitin-protein ligase activity
DNA repair	Microfibril	Iron ion binding
ESE vs mild endometriosis ESE		
Transport	Nucleus	Protein binding
Transcription	Extracellular region	Nucleotide binding
Cell adhesion	Cytoplasm	DNA binding
Proteolysis	Membrane	Nucleic acid binding
Cell cycle	Intracellular	Catalytic activity
Signal transduction	Mitochondrion	Receptor activity
Translation	Plasma membrane	Actin binding
Lipid metabolic process	Membrane fraction	Signal transducer activity
Skeletal system development	Endoplasmic reticulum	Structural molecule activity
Immune response	Golgi membrane	Transporter activity
Nuclear mRNA splicing, via spliceosome	Integral to plasma membrane	Calcium ion binding
Carbohydrate metabolic process	Lysosome	Cytokine activity
Apoptosis	Extracellular space	RNA binding
Ubiquitin-dependent protein catabolic process	Cytosol	Ion channel activity
Protein amino acid phosphorylation	Golgi apparatus	Magnesium ion binding
Metabolic process	Soluble fraction	Binding
Protein folding	Ubiquitin ligase complex	Structural constituent of ribosome
Protein modification process	Nucleosome	Zinc ion binding
Regulation of cell growth	Endosome	Receptor binding
rRNA processing	Chromatin	Hydrolase activity
tRNA processing	Ruffle	Endopeptidase inhibitor activity
Ossification	Mediator complex	Metalloendopeptidase activity
DNA repair	Cytoskeleton	Ubiquitin-protein ligase activity
Acute-phase response	Integral to membrane of membrane fraction	Phosphoprotein phosphatase activity
Regulation of transcription, DNA-dependent	Exosome (RNase complex)	Antigen binding
MSE vs mild endometriosis MSE		
Transcription	Nucleus	Nucleotide binding
Transport	Cytoplasm	Protein binding

(continued)

Table 2 (continued)

GO Biological Process	GO Cellular Component	GO Molecular Function
Signal transduction	Extracellular region	DNA binding
Cell adhesion	Intracellular	Nucleic acid binding
Translation	Membrane	Receptor activity
Nuclear mRNA splicing, via spliceosome	Mitochondrion	Catalytic activity
Proteolysis	Plasma membrane	Signal transducer activity
Lipid metabolic process	Endoplasmic reticulum	Binding
Protein amino acid phosphorylation	Membrane fraction	Actin binding
Ubiquitin-dependent protein catabolic process	Golgi membrane	Transporter activity
Negative regulation of transcription from RNA polymerase II promoter	Integral to plasma membrane	RNA binding
Cell cycle	Lysosome	Calcium ion binding
Multicellular organismal development	Golgi apparatus	Structural molecule activity
DNA repair	Cytosol	Magnesium ion binding
Angiogenesis	Soluble fraction	Structural constituent of ribosome
Immune response	Integral to membrane	Zinc ion binding
Skeletal system development	Ubiquitin ligase complex	Ion channel activity
Protein folding	Proteasome complex	Ubiquitin-protein ligase activity
Apoptosis	Nucleosome	Iron ion binding
Carbohydrate metabolic process	Ruffle	Endopeptidase inhibitor activity
Metabolic process	Endosome	Phosphoprotein phosphatase activity
Regulation of cell growth	Chromatin	Receptor binding
Protein modification process	Telomeric region	Iron ion transmembrane transporter activity
Regulation of transcription, DNA-dependent mRNA processing	Mitochondrion	Enzyme inhibitor activity
	Coated pit	hydrolase activity

Abbreviations: PE, proliferative endometrium; ESE, early secretory endometrium; MSE, mid-secretory endometrium.

membranes (Table 2), demonstrating the ubiquitous participation of all cellular components in molecular differences between severe and mild endometriosis. The main GO molecular function categories included nucleotide binding, protein and DNA binding, receptor activity, actin binding, signal transducer activity, and others.

Microarray Validation by Real-Time RT-PCR

Some of the highly up- or downregulated genes, as well as genes dysregulated in all cycle phases between severe and mild endometriosis groups were selected randomly for validation using real-time RT-PCR: *DIO2*, *IGFBP5*, *VCAN*, *SLC1A1*, *SST*, *TAGLN*, and *EGFR* (Figure 2; Supplemental Tables 1-3). Most of the validated genes (*VCAN*, *IGFBP5*, *SST*, *DIO2*) follow the trend differences of the microarray results.

Analysis of Networks and Canonical Pathways Regulated in Severe Versus Mild Endometriosis

Ingenuity pathway analysis of gene expression profiles revealed several associated network functions identified as different between severe versus mild endometriosis in proliferative, early secretory, and mid-secretory phase samples (Table 3). As expected, several genes are involved in more than 1 network/pathway. Comparison of canonical pathways regulated in

severe versus mild endometriosis revealed large differences in eutopic endometrium in these 2 disease stages, as shown by the high number of regulated genes in the pathways. The major canonical pathways regulated are presented in Table 4.

Major differences in neuregulin signaling, which involves members of the EGFR family, were observed in the proliferative and mid-secretory phases between severe versus mild endometriosis (Figure 3). Epidermal growth factor receptor mRNA was upregulated in severe versus mild endometriosis in PE and ESE (Figure 2B), indicating its involvement in severe disease, and confirming our earlier reports of the involvement of EGF family in the pathophysiology of severe endometriosis.^{13,24}

Epidermal Growth Factor Receptor Protein Immunoreactivity

As presented in Figure 4 and Table 5, EGFR protein was expressed throughout the menstrual cycle in women with mild as well as severe endometriosis. Interestingly, the most dramatic difference in EGFR protein immunoreactivity was observed in the early secretory phase, where strong stromal expression was observed in severe compared to mild endometriosis, consistent with the real-time RT-PCR data (Figure 2B, Figure 4E,H, Table 5). There was a slight increase in epithelial EGFR immunostaining in the proliferative phase of severe endometriosis samples (Figure 4D,G, Table 5), whereas

Table 3. Top Networks Regulated in Endometrium from Severe Versus Mild Endometriosis

Top 5 networks regulated in proliferative phase endometrium (PE) from severe endometriosis versus mild PE					
ID	Molecules in Network	Score	Focus Molecules	Top Functions	
1	ADAMTS9, Caspase, CDKN2A, Cyclin A, DBP, E2f, EN2, ERCC1, FOXL2, GAS2LI, GNAI1, GSPT1, GTF2H4, GTSE1, HNRNPA2B1, HNRNP1, Ifn gamma, IFNGR1, IL32, KRT17, LIN37, MAFF, MAP3K7IP2, NFkB (complex), NLRP1, PYCARD, RBCK1, RRAS, RRM1 (includes EG:6240), SUZ12, TANK, TFDPI, TFIH, UHMK1, WTAP	52	29	Cell Death, Hair and Skin Development and Function, Organ Development	
2	ANXA1, BAX, C12ORF10, Calpain, CBLC, DDX3X, DNAJA1, EGFR, FANCA, Fibrinogen, GNRH, HSP, Hsp70, Hsp90, HSP90AA1, HSP90AB1, HSP90B1, HSPA8, HSPH1, IFN Beta, IL1, LAMBI, LARPI, LRRFIPI, MACFI, MYOF, PAFAH1B3, PCMI, PI3K, PLA2, Proteasome, REEP6, SFRPI, SSB, YWHAZ	39	24	Cellular Compromise, Post-Translational Modification, Protein Folding	
3	I4-3-3, ADD3, Akt, ASAH1, BAD, CCNLI, Cdc2, CEL, CP, Ctbp, CTBPI, Cyclin E, DCN, DDX42, EXOSC4, FZD2, GADD45GIP1, HISTONE, HMG20B, HNRNPR, JDP2, MAP2K1/2, NFIC, p70 S6k, PDGF BB, PDPK1, PNN, PP2A, RBM5, RBMX, RPL13, SF3A2, SF3B1, SFRS1, SFRS7	39	25	RNA Post-Transcriptional Modification, Lipid Metabolism, Small Molecule Biochemistry	
4	ATP2B4, CIQTNF2, CTTN, DIO2, EPHA2, ERK, FAK, FBNI, Fgf, Fgfr, FGFR3, FOSL2, GDI2, IL27RA, ITGB1, JAK, JAK1, KLF13, LMO4, NRTN, NUFIP1, Pak, Pdgf, Pdgfr, PI3K p85, PLC gamma, PSMD7, PTPN11, RAB1A, Raf, RLIM, Shc, STAT, STAT5a/b, VCAN	32	21	Cellular Development, Skeletal and Muscular System Development and Function, Cellular Movement	
5	ARAP1, BRD4, Calmodulin, Ck2, CRIM1, CSDC2, GGA1, Histone h3, Ikb, IKK (complex), Insulin, MATR3, MIR21 (includes EG:406991), MRPL12, MSX2, MTUS1, MYCN, NOP2, NUDT1, PIK3R1, PSPC1, PTEN, RBM14, RNA polymerase II, RPL13, SFPQ, SLC39A4, SMC4, SORD, UBE2G2, UBE2I, Ubiquitin, XIST, ZNF146, ZNF451	27	19	Cell Cycle, Embryonic Development, Cancer	
Top 5 networks regulated in early secretory phase endometrium (ESE) from severe endometriosis versus mild ESE					
ID	Molecules in Network	Score	Focus Molecules	Top Functions	
1	ADAMTS9, APIS1, BCR, BEX2, BGN, CIq, CIQA, CIQB, CIS, CARD10, Complement component I, CXCL16, ENPPI, FMOD, G0S2, IgG, IGKC, IGL@, Igm, IL32, KRT7, KRT13, KRT17, NFkB (complex), NFKBIZ, PIGR, PTPLAD1, RBCK1, SERINC3, SERPING1, SLC16A1, SLC3A1, SLC7A1, STAP2, TNFRSF18	42	29	Skeletal and Muscular System Development and Function, Amino Acid Metabolism, Dermatological Diseases	
2	ALP, ALPP, ASS1, CCNO, CFD, DDX3X, DIO2, DKK1, DNMT3A, Fgf, FGF18, FOXL2, FOXS1, Frizzled, FRZB, FXYD5, FZD2, FZD8, FZD10, GASI, HES1, MAFF, MIB2, MSX2, MUC4, NEDD4L, P38 MAPK, PDGF BB, PORCN, SLC30A5, SMAD6, SOX4, TOBI, Wnt, WNT4	42	29	Cell Development, Connective Tissue Development and Function, Skeletal and Muscular System Development	
3	ADD3, AGTRAP, ATP1A2, BMP7, CIQTNF2, CCL5, CTSG, ERK, Fibrin, FXYD4, Growth hormone, Igf, Igfbp, IGFBP3, IGFBP5, IGFBP6, IL27RA, LCN2, Mmp, MMP7, Na ⁺ ,K ⁺ -ATPase, NADPH oxidase, NAMPT, NRTN, POSTN, PRPF4, RARRES2, SERPINA1, SERPINA3, Smad2/3-Smad4, STRA13, Tgf beta, TIMP1, VCAN, VitaminD3-VDR-RXR	30	24	Cellular Movement, Cancer, Gastrointestinal Disease	

(continued)

Table 3 (continued)

4	ABPI, Adaptor protein 2, ADRB2, ALDOA, Angiotensin II receptor type 1, Beta Arrestin, C12ORF10, CAV1, Clathrin, CMTM8, Creatine Kinase, DAB2, Dynamin, EGFR, FBPI, GFER, HDL, HSP90ABI, HSPA8, MACFI, MAT2A, Mek, MIR21 (includes EG:406991), NCK, NFIB, NUMA1, PLTP, PTPRS (includes EG:5802), SNX9 (includes EG:51429), Sos, SPDEF, TRAK1, TRAK2, TRH, Vegf	30	24	Cellular Assembly and Organization, Cardiac Hypertrophy, Cardiovascular Disease
5	ADCYAP1R1, AGR2, BLVRB, Cbp/p300, CYP26A1, DHRS13, FJX1, FOXO1, hCG, Histone h3, Histone h4, HMOX1, HOXB8, KLF6, LBH, LSR, MAF, MGMT, MTUS1, Nfat (family), NPTX2, NPTXR, Oxidoreductase, PDGFA, Pkc(s), PURA, Rxr, SFRP4, SP3, TAGLN, TBLIX, Thyroid hormone receptor, TNFRSF4, TSPO, XIST	30	26	Developmental Disorder, Genetic Disorder, Neurological Disease

Top 5 networks regulated in mid-secretory phase endometrium (MSE) from severe endometriosis versus mild MSE

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	AHNAK, ARID5B, B3GNT1, BGN, CAND2, CHI3L1, COL16A1, COL6A2, EHD2, ELN, FOXS1, FXVD6, HIFX, HNRPD, KCNG1, KRT7, MFAP2, MPHOSPH9, MYOF, NPTX2, NPTXR, PDLIM4, PDZK1IP1, PKIG, PLOD2, PMEPA1, RAB25, RAMP2, RBPMS, SCG5, SPAG4, STARD10, TGFBI, WFS1, ZNF581	51	35	Cancer, Cell-To-Cell Signaling and Interaction, Skeletal and Muscular Disorders
2	ANXA11, APBA3, APEX2, AQPI, BIK, C12ORF10, CBLC, CCT2, DOCK5, EFEMP2, EGFR, FAM107A, GAS5, GPC1, HNRNP1, HSP90ABI, HSPH1, LDOC1, LRRFIP1, MACFI, MBNLI, MPG, PAFAH1B3, PGK1, PHLDA1, PSMD7, PTPRS (includes EG:5802), RAB1A, RABAC1, SCAMPI, SFRP1, TRAK2, TUBB2A, VWWPI, ZNF638	51	35	Cardiovascular System Development and Function, Cellular Development, Cell Growth and Proliferation
3	BCLAF1, C19ORF43, CAMK2N1, CCNL2, CHD1 (includes EG:1105), CLK1, DDX42, DEAF1, ERAL1, ERK, GNRH, HMG20B, HNRNPA2B1, HNRNPL, IL27RA, IL6ST, KLF13, KLHL22, LMO4, LOXLI, PRPF4, RBM5, RBMX, RLIM, SEMA3F, SF3A2, SF3B1, SFRS1, SFRS5, SFRS7, SFRS11, SFRS2IP, TRA2A, UBXN1, VWSX1-gp130	42	32	RNA Post-Transcriptional Modification, Cancer, Cellular Growth and Proliferation
4	3 BETA HSD, AKR7A2, CCNLI, CNNI, CRIMI, CSRP2, DDX5, DUSP1, GLIS2, HELZ, HOXB8, HSD3B7, MAT2A, MEIS1, MIR21 (includes EG:406991), NAMPT, NBLI, NKIRAS2, PDGF BB, RNA polymerase II, RPL13, SERPINA3, SLC1A1, SP2, TAGLN, TEAD2, TEAD4, TIA1, TNCCI, TOB1, UNC5B, XAB2, XDH, YAPI, ZNF83	42	32	Cellular Assembly and Organization, Drug Metabolism, Genetic Disorder
5	ABCG1, ADAMTS9, AEBPI, ATF5, CARD10, CLIP2, CRISP3 (includes EG:10321), CXCL16, DBP, GFER, HDL, HES1, HEY2, LCN2, NCOA7, NFkB (complex), NFKBIZ, Notch, NOTCH2, NOTCH3, PLTP, PRDX2, PYCARD, RBCK1, RIOK3, RTKN, RTN4R, S100P, Secretase gamma, SLC3A1, SLC7A1, TAX1BP3, TCEA2, WTAP, ZMYND11	40	31	Amino Acid Metabolism, Molecular Transport, Small Molecule Biochemistry

immunostaining was similar in MSE samples, regardless of the endometriosis stage (Figure 4F, I, Table 5). Immunohistochemical analysis of EGFR in endometrial samples from women without endometriosis throughout the menstrual cycle revealed

weak expression of this protein in epithelial and/or stromal compartments (in particular, in ESE stroma), compared to the endometrium from women with endometriosis (Figure 4A-C, Table 5).

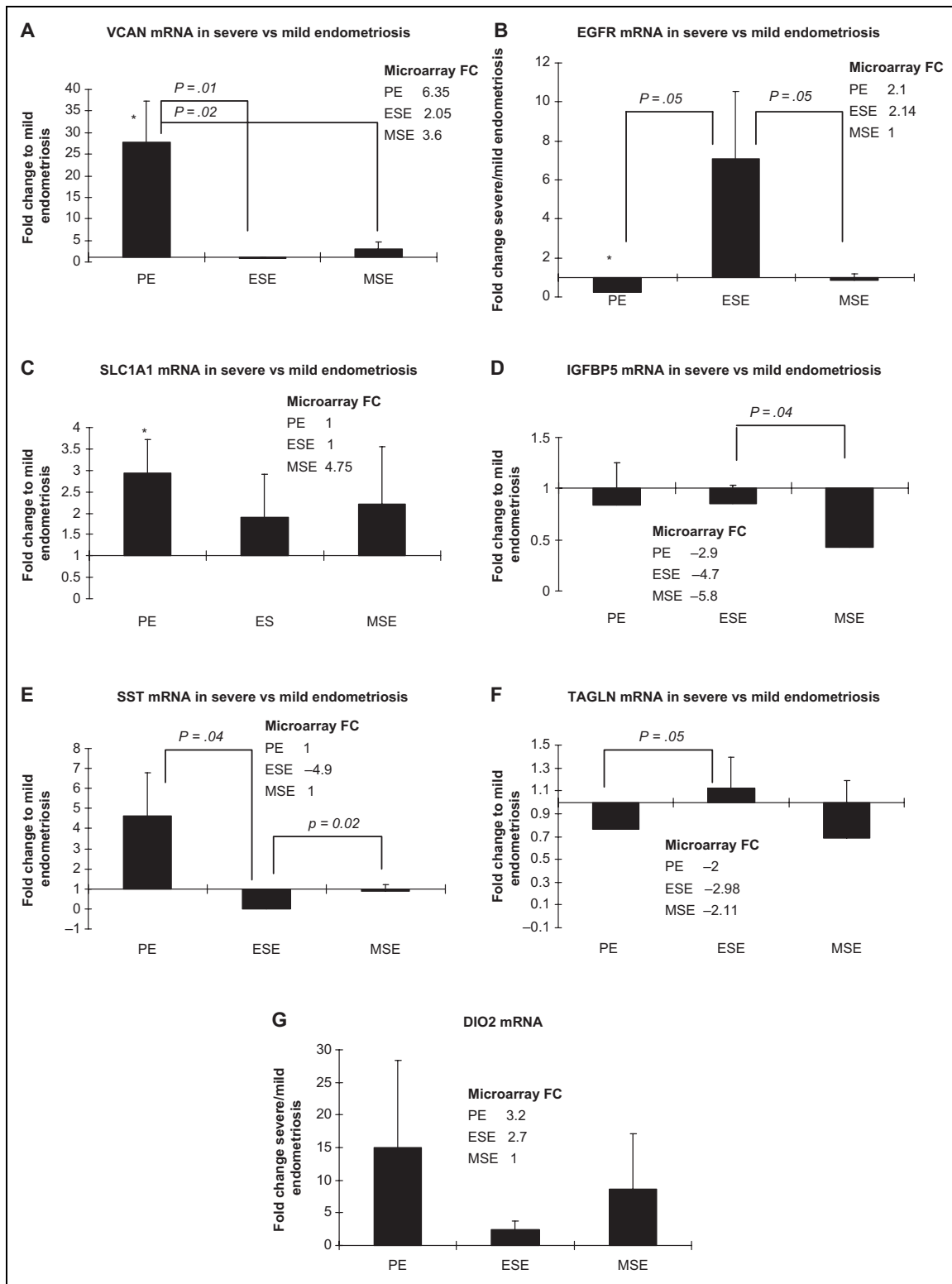


Figure 2. Quantitative real-time reverse transcriptase–polymerase chain reaction (QPCR) validation of microarray data. Panels A, VCAN; B, EGFR; C, SLC1A1; D, IGFBP5; E, SST; F, TAGLN; G, DIO2 gene expression in severe endometriosis expressed as fold change compared to the expression in mild endometriosis, throughout the menstrual cycle. Microarray data are presented in the insert. *Statistically significant differences ($P < .05$) between the same cycle phases in severe vs mild endometriosis determined by the (Mann-Whitney test). Error bars represent \pm SEM. PE indicates proliferative phase endometrium; ESE, early secretory phase endometrium; MSE, mid-secretory phase endometrium; SEM, standard error of the mean; VCAN, versican; EGFR, epidermal growth factor receptor; SLC1A1, solute carrier family 1, member 1; IGFBP5, insulin-like growth factor binding protein 5; SST, somatostatin; TAGLN, transgelin; DIO2, thyroxine deiodinase 2.

Table 4. Top 50 Canonical Pathways

Ingenuity Canonical Pathways	$-\log(P \text{ Value})$	$P \text{ Value}$	Ratio	Molecules
Pathways regulated in proliferative endometrium (PE) from severe endometriosis vs mild PE				
Neuregulin signaling	6.25E00	.000001	0.11	ITGB1, BAD, PTPN11, HSP90AB1, RRAS, PIK3R1, DCN, HSP90AA1, PDPK1, PTEN, EGFR
Prostate cancer signaling	4.83E00	.000015	0.09	BAD, TFDPI, PIK3C2A, HSP90AB1, RRAS, PIK3R1, HSP90AA1, PDPK1, PTEN
Non-small cell lung cancer signaling	4.64E00	.000023	0.10	CDKN2A, BAD, TFDPI, PIK3C2A, RRAS, PIK3R1, PDPK1, EGFR
PI3K/AKT signaling	4.35E00	.000045	0.07	ITGB1, JAK1, BAD, HSP90AB1, RRAS, PIK3R1, YWHAZ, HSP90AA1, PDPK1, PTEN
Chronic myeloid leukemia signaling	4.27E00	.000054	0.09	CDKN2A, CTBP1, BAD, TFDPI, PTPN11, PIK3C2A, RRAS, PIK3R1, CBLC
Myc-mediated apoptosis signaling	4.17E00	.000068	0.12	CDKN2A, BAD, PIK3C2A, RRAS, PIK3R1, YWHAZ, BAX
Melanoma signaling	3.98E00	.000105	0.13	CDKN2A, BAD, PIK3C2A, RRAS, PIK3R1, PTEN
IGF-1 signaling	3.69E00	.000204	0.08	BAD, PTPN11, PIK3C2A, RRAS, PIK3R1, YWHAZ, PDPK1, IGFBP5
P70S6K signaling	3.51E00	.000309	0.07	GNAI2, JAK1, BAD, PIK3C2A, RRAS, PIK3R1, YWHAZ, PDPK1, EGFR
Endometrial cancer signaling	3.45E00	.000355	0.11	BAD, PIK3C2A, RRAS, PIK3R1, PDPK1, PTEN
Docosahexaenoic acid (DHA) signaling	3.39E00	.000407	0.11	BAD, PIK3C2A, PIK3R1, PDPK1, BAX
FAK signaling	3.1E00	.000794	0.07	ITGB1, PIK3C2A, RRAS, PIK3R1, PDPK1, PTEN, EGFR
PTEN signaling	2.89E00	.001288	0.07	ITGB1, BAD, RRAS, PIK3R1, PDPK1, PTEN, EGFR
FLT3 signaling in hematopoietic progenitor cells	2.83E00	.001479	0.08	BAD, PTPN11, PIK3C2A, RRAS, PIK3R1, PDPK1
Glioma signaling	2.75E00	.001778	0.06	CDKN2A, TFDPI, PIK3C2A, RRAS, PIK3R1, PTEN, EGFR
CNTF signaling	2.75E00	.001778	0.09	JAK1, PTPN11, PIK3C2A, RRAS, PIK3R1
Insulin receptor signaling	2.68E00	.002089	0.06	JAK1, BAD, PTPN11, PIK3C2A, RRAS, PIK3R1, PDPK1, PTEN
Pancreatic adenocarcinoma signaling	2.6E00	.002512	0.06	CDKN2A, JAK1, BAD, TFDPI, PIK3C2A, PIK3R1, EGFR
IL-2 signaling	2.56E00	.002754	0.09	JAK1, PTPN11, PIK3C2A, RRAS, PIK3R1
Molecular mechanisms of cancer	2.49E00	.003236	0.04	CDKN2A, JAK1, PIK3C2A, BAD, TFDPI, RRAS, PIK3R1, MAP3K7IP2, GNAI1, BAX, APH1A (includes EG:51107), GNAI2, PTPN11, FZD2
FcγRIIB signaling in B lymphocytes	2.31E00	.004898	0.07	PIK3C2A, RRAS, PIK3R1, PDPK1
JAK/Stat signaling	2.3E00	.005012	0.08	JAK1, PTPN11, PIK3C2A, RRAS, PIK3R1
p53 signaling	2.29E00	.005129	0.07	CDKN2A, SCO2 (includes EG:9997), PIK3C2A, PIK3R1, BAX, PTEN
Glucocorticoid receptor signaling	2.28E00	.005248	0.04	HSPA8, JAK1, PIK3C2A, HSP90AB1, RRAS, GTF2H4, PIK3R1, ANXA1, HSP90AA1, CCL5, UBE2I
Angiopietin signaling	2.27E00	.005370	0.07	BAD, PTPN11, PIK3C2A, RRAS, PIK3R1
ERK5 signaling	2.24E00	.005754	0.07	BAD, PTPN11, RRAS, YWHAZ, EGFR
Neurotrophin/TRK signaling	2.18E00	.006607	0.06	PTPN11, PIK3C2A, RRAS, PIK3R1, PDPK1
IL-3 signaling	2.04E00	.009120	0.07	JAK1, BAD, PIK3C2A, RRAS, PIK3R1
Agrin interactions at neuromuscular junction	2.04E00	.009120	0.07	ITGB1, RRAS, LAMB1, CTTN, EGFR
EGF signaling	2.01E00	.009772	0.08	JAK1, PIK3C2A, PIK3R1, EGFR
Aryl hydrocarbon receptor signaling	1.89E00	.012882	0.04	CDKN2A, NFIC, TFDPI, HSP90AB1, HSP90AA1, GSTT2, BAX
I4-3-3-mediated signaling	1.86E00	.013804	0.05	BAD, PIK3C2A, RRAS, PIK3R1, YWHAZ, BAX
Aldosterone signaling in epithelial cells	1.83E00	.014791	0.05	HSPA8, PIK3C2A, PIK3R1, HSP90AA1, PDPK1
EIF2 signaling	1.79E00	.016218	0.05	PIK3C2A, RRAS, PIK3R1, EIF4G3, PDPK1
Thrombopoietin signaling	1.77E00	.016982	0.06	PTPN11, PIK3C2A, RRAS, PIK3R1

(continued)

Table 4 (continued)

Ingenuity Canonical Pathways	$-\log(P \text{ Value})$	$P \text{ Value}$	Ratio	Molecules
Mitotic roles of polo-like kinase	1.77E00	.016982	0.06	ANAPC4, HSP90AB1, HSP90AA1, CDC16
IL-9 signaling	1.73E00	.018621	0.08	JAK1, PIK3C2A, PIK3R1
Bladder cancer signaling	1.69E00	.020417	0.05	CDKN2A, FGFR3, TFDPI, RRAS, EGFR
Colorectal cancer metastasis signaling	1.68E00	.020893	0.04	JAK1, BAD, PIK3C2A, RRAS, PIK3R1, IFNGR1, BAX, FZD2, EGFR
G beta gamma signaling	1.63E00	.023442	0.04	GNAI2, RRAS, GNAI1, PDPK1, EGFR
IL-15 signaling	1.6E00	.025119	0.06	JAK1, PIK3C2A, RRAS, PIK3R1
Hypoxia signaling in the Cardiovascular system	1.58E00	.026303	0.06	HSP90AB1, HSP90AA1, PTEN, UBE2I
GM-CSF signaling	1.58E00	.026303	0.06	PTPN11, PIK3C2A, RRAS, PIK3R1
IL-4 signaling	1.53E00	.029512	0.06	JAK1, PIK3C2A, RRAS, PIK3R1
Macropinocytosis signaling	1.53E00	.029512	0.06	ITGB1, PIK3C2A, RRAS, PIK3R1
Erythropoietin signaling	1.51E00	.030903	0.05	PIK3C2A, RRAS, PIK3R1, PDPK1
Fc Epsilon RI signaling	1.51E00	.030903	0.05	PTPN11, PIK3C2A, RRAS, PIK3R1, PDPK1
Huntington disease signaling	1.48E00	.033113	0.03	HSPA8, PIK3C2A, PIK3R1, GNAI1, STX16, PDPK1, BAX, EGFR
HGF signaling	1.47E00	.033884	0.05	CDKN2A, PTPN11, PIK3C2A, RRAS, PIK3R1
iCOS-iCOSL signaling in T Helper Cells	1.46E00	.034674	0.04	BAD, PIK3C2A, PIK3R1, PDPK1, PTEN
Pathways regulated in early secretory endometrium (ESE) from severe endometriosis vs mild ESE				
Complement system	3.54E00	.00029	0.19	CFD, SERPING1, C1S, CD55, C1QA, CD46, C1QB
Hepatic fibrosis/hepatic stellate cell activation	2.92E00	.00120	0.10	VCAM1, FN1, PDGFA, IFNGR1, IGFBP5, BAX, CCL5, PGF, MYL9 (includes EG:10398), IGF1, TIMP1, IGFBP3, EGFR
IL-8 signaling	2.72E00	.00191	0.08	NAPEPLD, VCAM1, PIK3C2A, RRAS, PIK3R1, BAX, IRAK1, PGF, EIF4EBP1, ROCK2, HMOX1, CCND2, RHOD, RHOF, EGFR
Clathrin-mediated endocytosis signaling	2.71E00	.00195	0.08	ACTR2, PIK3C2A, PDGFA, PIK3R1, ITGB8, PGF, HSPA8, SNX9 (includes EG:51429), IGF1, FGF18, RAB5C, DAB2, CTTN, ITGB5
Docosahexaenoic acid (DHA) signaling	2.67E00	.00214	0.13	BAD, FOXO1, PIK3C2A, PIK3R1, BIK, BAX
IGF-1 signaling	2.65E00	.00224	0.10	IGFBP6, IGF1, BAD, FOXO1, PIK3C2A, RRAS, PIK3R1, IGFBP3, IGFBP5, SFN
Prostate cancer signaling	2.41E00	.00389	0.09	BAD, TFDPI, FOXO1, PIK3C2A, HSP90AB1, RRAS, SRD5A1, PIK3R1, CREB3L4
Myc-mediated apoptosis signaling	2.23E00	.00589	0.12	IGF1, BAD, PIK3C2A, RRAS, PIK3R1, BAX, SFN
NRF2-mediated oxidative stress response	2.1E00	.00794	0.08	AKR7A2, MGST1, PIK3C2A, RRAS, PIK3R1, MAF, CLPP, MAFF, HMOX1, DNAJC4, GSTT2, GSTO2, FKBP5, PTPLAD1
Ubiquinone biosynthesis	2.07E00	.00851	0.06	NDUFB11, NDUFS8, NDUFS7, MGMT, NDUFB7, NDUF3, ALDH6A1
IL-4 signaling	1.92E00	.01202	0.10	HLA-DQB1, SOCS1, HLA-DMA, JAK1, PIK3C2A, RRAS, PIK3R1
VEGF signaling	1.81E00	.01549	0.08	ROCK2, BAD, FOXO1, PIK3C2A, RRAS, PIK3R1, SFN, PGF
Pancreatic adenocarcinoma signaling	1.77E00	.01698	0.08	HMOX1, NAPEPLD, JAK1, BAD, TFDPI, PIK3C2A, PIK3R1, PGF, EGFR
Virus entry via endocytic pathways	1.75E00	.01778	0.08	PIK3C2A, RRAS, PIK3R1, CD55, CAV1, ITGB8, ITGB5, FOLR1
Caveolar-mediated endocytosis signaling	1.72E00	.01905	0.09	CD55, RAB5C, CAV1, COPE, ITGB8, ITGB5, EGFR
Axonal guidance signaling	1.65E00	.02239	0.06	ACTR2, FZD10, PIK3C2A, UNC5A, RRAS, PDGFA, PIK3R1, GNAI1, EFNA4, PGF, MYL9 (includes EG:10398), ROCK2, FZD8, IGF1, GLIS2, RHOD, NTRK3, WNT4, BMP7, EFNB3, RTN4R, FZD2, GLIS1
Role of NANOG in mammalian embryonic stem cell pluripotency	1.62E00	.02399	0.09	FZD8, FZD10, JAK1, PIK3C2A, RRAS, PIK3R1, WNT4, BMP7, TCF7L1, FZD2

(continued)

Table 4 (continued)

Ingenuity Canonical Pathways	−log(P Value)	P Value	Ratio	Molecules
Fructose and mannose metabolism	1.56E00	.02754	0.03	AKR7A2, GMDS, GALK1, FBPI, ALDOA
Colorectal cancer metastasis signaling	1.55E00	.02818	0.06	FZD10, MMP7, JAK1, PIK3C2A, BAD, RRAS, PIK3R1, IFNGRI, BAX, PGF, FZD8, RHOD, WNT4, RHOF, FZD2, EGFR
Interferon signaling	1.55E00	.02818	0.13	SOCS1, JAK1, IFITM1, IFNGRI
Glucocorticoid receptor signaling	1.52E00	.03020	0.06	VCAM1, JAK1, PIK3C2A, RRAS, PIK3R1, CCL5, SLPI, HSPA8, HSP90ABI, DUSP1, GTF2H4, CDKN1C, FKBP5, POLR2I, UBE2I, ADRB2
VDR/RXR activation	1.52E00	.03020	0.09	IGFBP6, FOXO1, PDGFA, IGFBP3, IGFBP5, HES1, CCL5
ILK signaling	1.51E00	.03090	0.06	MYL9 (includes EG:10398), PARVB, FNI, PIK3C2A, RHOD, PIK3R1, CREB3L4, ITGB8, RHOF, PPP1R14B, ITGB5, PGF
Basal cell carcinoma signaling	1.47E00	.03388	0.10	FZD8, FZD10, GLIS2, WNT4, BMP7, FZD2, GLIS1
PI3K/AKT signaling	1.46E00	.03467	0.07	JAK1, BAD, FOXO1, HSP90ABI, RRAS, PIK3R1, SFN, EIF4EBP1, THEM4
Human embryonic stem cell pluripotency	1.44E00	.03631	0.07	FZD8, FZD10, PIK3C2A, PDGFA, NTRK3, PIK3R1, SMAD6, WNT4, BMP7, FZD2
Wnt/β-catenin signaling	1.44E00	.03631	0.07	SOX4, FZD8, SFRP4, FZD10, MMP7, FRZB, CDH3, WNT4, TCF7L1, PIN1, DKK1, FZD2
Macropinocytosis signaling	1.42E00	.03802	0.08	PIK3C2A, RRAS, PDGFA, PIK3R1, ITGB8, ITGB5
Non-small cell lung cancer signaling	1.39E00	.04074	0.08	BAD, TFDPI, PIK3C2A, RRAS, PIK3R1, EGFR
Riboflavin metabolism	1.39E00	.04074	0.05	ACP5, ENPP3, ENPPI
FLT3 signaling in hematopoietic progenitor cells	1.31E00	.04898	0.08	BAD, PIK3C2A, RRAS, PIK3R1, CREB3L4, EIF4EBP1
Glutathione metabolism	1.29E00	.05129	0.05	GPX3, MGST1, GPX1, GSTT2, GSTO2
Selenoamino acid metabolism	1.28E00	.05248	0.04	MGMT, PAPSS1 (includes EG:9061), MAT2A
p53 signaling	1.27E00	.05370	0.08	SCO2 (includes EG:9997), CCND2, PIK3C2A, PIK3R1, RPRM, BAX, SFN
IL-2 signaling	1.26E00	.05495	0.09	SOCS1, JAK1, PIK3C2A, RRAS, PIK3R1
IL-3 signaling	1.26E00	.05495	0.08	JAK1, BAD, FOXO1, PIK3C2A, RRAS, PIK3R1
Amyotrophic lateral sclerosis signaling	1.25E00	.05623	0.06	IGFI, PIK3C2A, PIK3R1, RAB5C, GPX1, BAX, PGF
I4-3-3-mediated signaling	1.24E00	.05754	0.07	BAD, FOXO1, PIK3C2A, RRAS, PIK3R1, BAX, SFN, PLCLI
PDGF signaling	1.23E00	.05888	0.08	JAK1, PIK3C2A, RRAS, PDGFA, PIK3R1, CAVI
Acute phase response signaling	1.22E00	.06026	0.06	HMOX1, SOCS1, SERPING1, FNI, RBP7, RRAS, CIS, PIK3R1, SERPINA3, SERPINA1, IRAK1
Integrin signaling	1.21E00	.06166	0.06	ACTR2, PARVB, PIK3C2A, RHOD, RRAS, PIK3R1, CAVI, ITGB8, RHOF, TSPAN4, ITGB5, RAP2A
Nitric oxide signaling in the cardiovascular system	1.21E00	.06166	0.07	PIK3C2A, HSP90ABI, PIK3R1, CAVI, SLC7A1, PGF
Molecular mechanisms of cancer	1.21E00	.06166	0.05	FZD10, JAK1, PIK3C2A, BAD, TFDPI, RRAS, PIK3R1, GNA11, SMAD6, BAX, APH1A (includes EG:51107), FZD8, CCND2, FOXO1, RHOD, IHH, BMP7, RHOF, FZD2, RAP2A
HMGB1 signaling	1.19E00	.06457	0.07	VCAM1, PIK3C2A, RHOD, RRAS, PIK3R1, IFNGRI, RHOF
Nicotinate and nicotinamide metabolism	1.17E00	.06761	0.05	ENPP3, PRKX, ENPPI, QPRT, NAMPT, HIPK1, IRAK1
Leukocyte extravasation signaling	1.14E00	.07244	0.06	ROCK2, CLDN10, MMP7, VCAM1, PIK3C2A, ICAM3, TIMP1, PIK3R1, RDX, MLLT4, CTTN
Glioma signaling	1.11E00	.07762	0.06	IGFI, TFDPI, PIK3C2A, RRAS, PDGFA, PIK3R1, EGFR
Methionine metabolism	1.1E00	.07943	0.04	DNMT3A, IL4I1, MAT2A
mTOR signaling	1.1E00	.07943	0.06	HMOX1, NAPEPLD, PIK3C2A, RHOD, RRAS, PIK3R1, RHOF, PGF, EIF4EBP1
Melanoma signaling	1.1E00	.07943	0.09	BAD, PIK3C2A, RRAS, PIK3R1

(continued)

Table 4 (continued)

Ingenuity Canonical Pathways	−log(P Value)	P Value	Ratio	Molecules
Pathways regulated in mid-secretory endometrium (MSE) from severe endometriosis vs mild MSE				
Neuregulin signaling	3.13E00	.00074	0.14	ITGB1, BAD, PIK3R1, DCN, PDPK1, PTEN, ERBB2IP (includes EG:55914), PRKCI, HSP90AB1, PTPN11, CDK5, HSP90AA1, PSEN1, EGFR
Acute phase response signaling	2.63E00	.00234	0.11	IL6ST, SOCS1, MAP2K7, CIS, PIK3R1, PDPK1, CP, SERPINA3, MAP3K5 (includes EG:4217), IL1RI, TCF3, IRAK1, HMOX1, SOD2, RIPK1, PTPN11, C4BPA, MAP3K7, CRABP2, SERPINA1
Docosahexaenoic acid (DHA) SIGNALING	2.37E00	.00427	0.16	BAD, FOXO1, PIK3C2A, PIK3R1, BIK, PDPK1, BAX
Wnt/β-catenin signaling	2.34E00	.00457	0.12	SOX4, SOX7, FZD10, FRAT1, TCF7L1, TCF3, SOX17, CSNK1E, FZD8, TGFB1, MAP3K7, DKK3, CD44, PIN1, SOX18, SFRP1, DKK1, FZD2, FZD7
Germ cell-sertoli cell junction signaling	2.14E00	.00724	0.11	EPN3, ITGB1, MAP2K7, PIK3C2A, CDC42, TJPI, PIK3R1, TUBG1, TUBB2A, PDPK1, MAP3K5 (includes EG:4217), IQGAPI, GSN, RHOQ, TGFB1, MAP3K7, PPAP2B, ACTN1
Clathrin-mediated endocytosis signaling	2.11E00	.00776	0.10	ITGB1, ACTR2, PIK3C2A, RAB5A, CDC42, PDGFA, PIK3R1, CLTB, HSPA8, MET, ARRB1, FGF18, RAB5C, DAB2, TFRC, CTTN, ITGB5
Prostate cancer signaling	2.09E00	.00813	0.12	BAD, TFDPI, FOXO1, PIK3C2A, HSP90AB1, SRD5A1, PIK3R1, HSP90AA1, PDPK1, CREB3L4, PTEN
Mitochondrial dysfunction	1.94E00	.01148	0.09	NDUFS7, XDH, NDUFA13, APH1A (includes EG:51107), MAOB, NDUFB11, SOD2, NDUFS8, TXN2, NDUFB7, NDUFA3, CYC1, UQCRC1, PSEN1, MAOA
Virus entry via endocytic pathways	1.89E00	.01288	0.12	ITGB1, PRKCI, CDC42, PIK3C2A, PIK3R1, CLTB, CD55, CAV1, TFRC, ITGB5, FOLR1
Macropinocytosis signaling	1.86E00	.01380	0.13	ITGB1, MET, PRKCI, RAB5A, CDC42, PIK3C2A, PDGFA, PIK3R1, ITGB5
Leukocyte extravasation signaling	1.83E00	.01479	0.09	ITGB1, PIK3C2A, CDC42, PIK3R1, RDX, THY1, ROCK2, GNAI2, CLDN23, PRKCI, PTPN11, ICAM3, EZR, CD44, MMP11, CTTN, ACTN1, TIMP2
VDR/RXR activation	1.78E00	.01660	0.13	IGFBP6, SPP1, PRKCI, FOXO1, PDGFA, IGFBP5, NCOR2, HES1, CCL5, KLF4
Notch signaling	1.77E00	.01698	0.16	RFNG, NOTCH2, NOTCH3, HEY2, HES1, APH1A (includes EG:51107), PSEN1
NRF2-mediated oxidative stress response	1.5E00	.03162	0.09	AKR7A2, MAP2K7, PIK3C2A, PIK3R1, SLC35A2, DNAJC13, DNAJC3, MAP3K5 (includes EG:4217), DNAJAI, CLPP, MAFF, MAFG, HMOX1, PRKCI, SOD2, DNAJC4, MAP3K7
Huntington disease signaling	1.43E00	.03715	0.08	MAP2K7, CAPN6, PIK3C2A, PIK3R1, GNAI1, HSPA9, PDPK1, CREB3L4, BAX, GNG7, HDAC5, HSPA8, PRKCI, GNG11, CDK5, STX16, NCOR2, POLR2I, EGFR
Hepatic fibrosis/hepatic stellate cell activation	1.42E00	.03802	0.10	PDGFA, FGFR1, FGFR2, IGFBP5, IL1RI, BAX, CCL5, MET, COL1A2, TGFB1, COL3A1, TIMP2, EGFR
Insulin receptor signaling	1.42E00	.03802	0.09	JAK1, BAD, PIK3C2A, PIK3R1, PDPK1, VAMP2, PTEN, EIF4EBP1, PRKCI, RHOQ, FOXO1, PTPN11, PPP1R12A
IGF-I signaling	1.42E00	.03802	0.10	IGFBP6, PRKCI, BAD, FOXO1, PTPN11, PIK3C2A, PIK3R1, YWHAZ, PDPK1, IGFBP5
Neurotrophin/TRK signaling	1.39E00	.04074	0.10	MAP2K7, CDC42, PTPN11, PIK3C2A, PIK3R1, PDPK1, CREB3L4, MAP3K5 (includes EG:4217)
G beta gamma signaling	1.39E00	.04074	0.09	GNAI2, GNAS, GNG11, PRKCI, CDC42, GNAI1, CAV1, PDPK1, GNG7, EGFR

(continued)

Table 4 (continued)

Ingenuity Canonical Pathways	−log(P Value)	P Value	Ratio	Molecules
PI3K/AKT signaling	1.38E00	.04169	0.09	ITGB1, JAK1, BAD, FOXO1, HSP90AB1, PIK3R1, YWHAZ, HSP90AA1, PDPK1, MAP3K5 (includes EG:4217), PTEN, EIF4EBP1
Semaphorin signaling in neurons	1.38E00	.04169	0.13	ROCK2, ITGB1, MET, RHOQ, CDK5, DPYSL4, NRPI
Mitotic roles of polo-like kinase	1.38E00	.04169	0.11	ANAPC4, HSP90AB1, TGFB1, CDC7, HSP90AA1, CDC16, STAG2
Histidine metabolism	1.37E00	.04266	0.05	PRPS2, ALDH3B2 (includes EG:222), MAOB, MGMT, ABPI, MAOA
SAPK/JNK signaling	1.36E00	.04365	0.10	MAP2K7, GNG11, RIPK1, TRD@, CDC42, PIK3C2A, MAP3K7, PIK3R1, MAP3K5 (includes EG:4217), GNG7
Type I diabetes mellitus signaling	1.34E00	.04571	0.10	SOCS1, HLA-DMA, MAP2K7, JAK1, RIPK1, TRD@, MAP3K7, IL1R1, MAP3K5 (includes EG:4217), HSPDI, IRAK1
FGF signaling	1.3E00	.05012	0.10	MET, PTPN11, PIK3C2A, FGF18, PIK3R1, FGFR1, FGFR2, CREB3L4, MAP3K5 (includes EG:4217)
Chronic myeloid leukemia signaling	1.25E00	.05623	0.10	CTBP1, RBL2, BAD, TFDPI, PTPN11, PIK3C2A, TGFB1, PIK3R1, CBLC, HDAC5
RAR activation	1.25E00	.05623	0.08	CYP26A1, PIK3R1, NR2F2, PDPK1, MAP3K5 (includes EG:4217), PTEN, PRKCI, DUSP1, GTF2H4, TGFB1, CRABP2, RDH5, NCOR2, CARM1, SCAND1
Phenylalanine metabolism	1.25E00	.05623	0.05	ALDH3B2 (includes EG:222), MAOB, ABPI, MAOA, PRDX2
14-3-3-mediated signaling	1.24E00	.05754	0.10	PRKCI, BAD, FOXO1, PIK3C2A, PIK3R1, TUBB2A, TUBG1, YAPI, YWHAZ, BAX, MAP3K5 (includes EG:4217)
Caveolar-mediated endocytosis signaling	1.23E00	.05888	0.10	ITGB1, RAB5A, CD55, RAB5C, CAV1, COPE, ITGB5, EGFR
Ubiquinone biosynthesis	1.17E00	.06761	0.06	NDUFB11, NDUFS8, NDUFS7, MGMT, NDUFB7, NDUFA3, NDUFA13
RAN signaling	1.17E00	.06761	0.13	KPNB1, RANBP2, RAN
ILK signaling	1.11E00	.07762	0.08	MUC1, ITGB1, PIK3C2A, CDC42, PIK3R1, FERMT2, PDPK1, CREB3L4, PTEN, PARVB, RHOQ, PPAP2B, CHD1 (includes EG:1105), ACTN1, ITGB5
Human embryonic stem cell pluripotency	1.07E00	.08511	0.08	FZD8, GNAS, FZD10, PIK3C2A, PDGFA, TGFB1, PIK3R1, FGFR1, FGFR2, PDPK1, FZD2, FZD7
B Cell receptor signaling	1.06E00	.08710	0.08	MAP2K7, BAD, CDC42, PIK3C2A, PIK3R1, CREB3L4, MAP3K5 (includes EG:4217), PTEN, PTPRC, PTPN11, CARD10, MAP3K7, PAG1
Ephrin receptor signaling	1.04E00	.09120	0.08	ITGB1, ACTR2, CDC42, PDGFA, GNAI1, CREB3L4, GNG7, EFNA4, ROCK2, GNAI2, EPHB6, GNAS, GNG11, PTPN11, EFNB3
Pentose phosphate pathway	9.86E-01	.10328	0.04	PRPS2, PGLS, FBPI, ALDOA
Melanoma signaling	9.75E-01	.10593	0.11	BAD, PIK3C2A, PIK3R1, CHD1 (includes EG:1105), PTEN
CCR5 signaling in macrophages	9.7E-01	.10715	0.08	GNAI2, GNAS, GNG11, PRKCI, TRD@, CCL5, GNG7
FLT3 signaling in hematopoietic progenitor cells	9.45E-01	.11350	0.10	BAD, PTPN11, PIK3C2A, PIK3R1, PDPK1, CREB3L4, EIF4EBP1
Oxidative phosphorylation	9.28E-01	.11803	0.08	ATP6V0E2, ATP6V0B, ATP5D, NDUFS7, TCIRG1, ATP6V1A, NDUFA13, NDUFB11, NDUFS8, NDUFB7, NDUFA3, CYC1, UQCRC1
IL-8 signaling	9.27E-01	.11830	0.07	PIK3C2A, PIK3R1, BAX, GNG7, EIF4EBP1, IRAK1, ROCK2, GNAI2, HMOX1, GNAS, GNG11, PRKCI, RHOQ, EGFR
Fructose and mannose metabolism	9.1E-01	.12303	0.04	AKR7A2, TSTA3, GMPPA, FBPI, ALDOA, FUK

(continued)

Table 4 (continued)

Ingenuity Canonical Pathways	−log(P Value)	P Value	Ratio	Molecules
Cdc42 signaling	9E-01	.12589	0.08	ITGB1, ANAPC4, ACTR2, PRKCI, TRD@, CDC42, CDC42EP5, PPP1R12A, CDC16, IQGAP1
Arginine and proline metabolism	8.96E-01	.12706	0.04	CKB, MAOB, VNN1, GAMT, LOXLI, ABPI, MAOA
HGF signaling	8.69E-01	.13521	0.09	MET, MAP2K7, PRKCI, CDC42, PTPN11, PIK3C2A, MAP3K7, PIK3R1, MAP3K5 (includes EG:4217)
EGF signaling	8.5E-01	.14125	0.10	MAP2K7, JAK1, PIK3C2A, PIK3R1, EGFR
Nitric oxide signaling in the cardiovascular system	8.49E-01	.14158	0.08	PIK3C2A, HSP90AB1, PIK3R1, CAVI, HSP90AA1, SLC7A1, CACNA1A

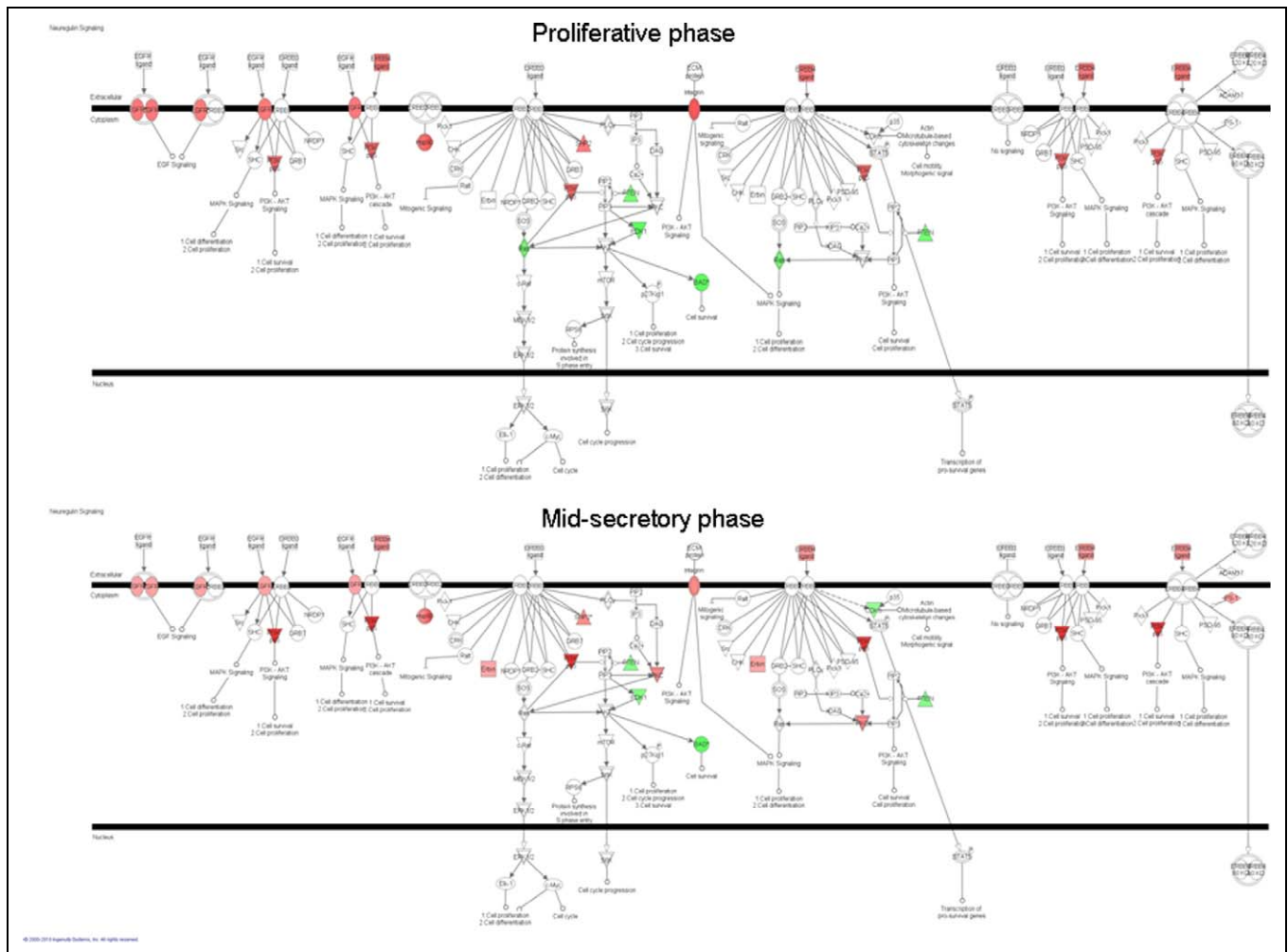


Figure 3. Neuregulin pathway regulation in proliferative endometrium (PE) and mid-secretory endometrium (MSE) from participants with severe vs mild endometriosis analyzed by ingenuity pathway analysis (IPA). Red color indicates upregulation of a gene; green color, down-regulation.

Versican Protein Immunoreactivity

In the proliferative phase, there was a strong stromal and epithelial VCAN immunostaining in severe and mild endometriosis, respectively (Figure 5D,G, Table 5). In ESE, similar VCAN immunostaining was observed in the stroma and

epithelial compartments (Figure 5E,H, Table 5), and epithelial VCAN immunostaining in MSE tended to be stronger in the severe endometriosis samples (Figure 5F,I, Table 5). Of note, diffuse stromal reactivity of VCAN in both cellular and extracellular compartments was observed. Remarkable was the VCAN immunoreactivity in the vasculature—in the smooth

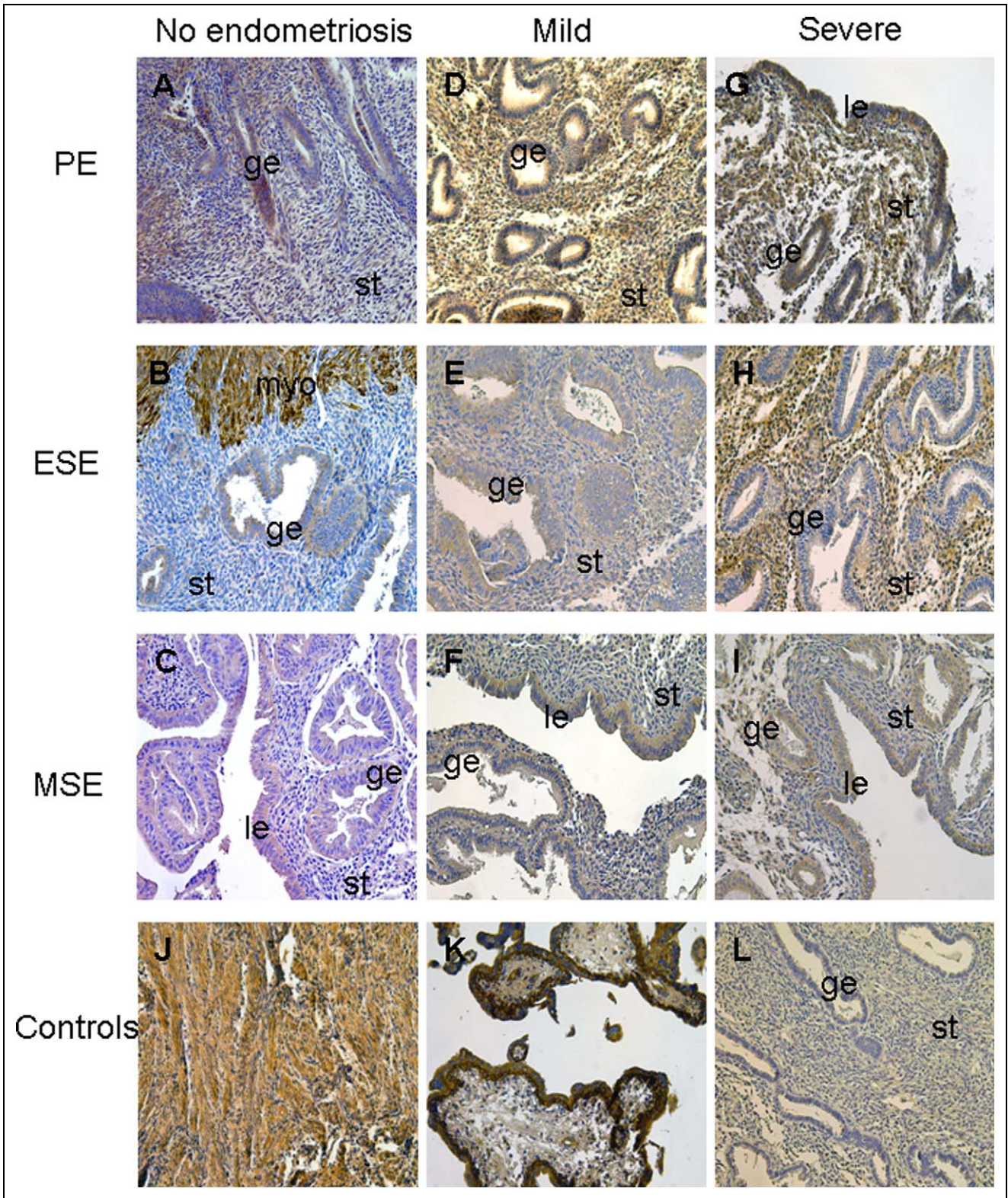


Figure 4. Epidermal growth factor receptor (EGFR) immunoreactivity in endometrial tissue from women without endometriosis (A-C), women with mild (D-F), and severe (G-I) endometriosis in the proliferative phase ([PE] n = 4, n = 4, and n = 5, respectively; A, D, G), early secretory phase ([ESE] n = 4, n = 3, and n = 7, respectively; B, E, H), and mid-secretory phase ([MSE] n = 4, n = 3, and n = 5, respectively; C, F, I) of the cycle. Human myometrial tissue was used as an internal positive control (on the same slide as endometrium, adjacent to the basalis endometrium, J). Human 12-week gestation placental tissue (K) was used as an additional positive control. L indicates negative control, nonimmune IgG-treated human endometrium; Le, luminal epithelium; ge, glandular epithelium; st, stroma; myo, myometrium; IgG, immunoglobulin G. Magnification $\times 200$.

Table 5. Semiquantitative Evaluation of VCAN and EGFR Immunostaining in Human Endometrial Tissue Sections From Women With Mild And Severe Endometriosis, As Well As Without Endometriosis

Protein	PE Mild Endometriosis		ESE Mild Endometriosis		MSE Mild Endometriosis		PE Severe Endometriosis		ESE Severe Endometriosis		MSE Severe Endometriosis	
	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma	Epithelium	stroma	Epithelium	Stroma
EGFR	+	++	+	+/>+++	++	++	++	++	+	+++	++	++
VCAN	-/+	+++	++	+	++	++	+++	+	++	+	+++	++

Protein	PE no endometriosis		ESE no endometriosis		MSE no endometriosis	
	Epithelium	Stroma	Epithelium	Stroma	Epithelium	Stroma
EGFR	++	++	+	+	++	+
VCAN	++	+	+/>+++	+	++	+

Abbreviations: PE, proliferative phase endometrium; ESE, early secretory phase endometrium; MSE, mid-secretory endometrium; VCAN, versican; EGFR, epidermal growth factor receptor; -, no staining, -/+, a few stained cells; +, faint staining; ++, moderate staining; +++, strong staining.

muscle layer and endothelial cells, regardless of disease status, stage, or cycle phase. Versican immunostaining in endometrial tissue from women without endometriosis throughout the menstrual cycle demonstrated overall weaker expression compared to samples from women with mild or severe endometriosis. This was particularly evident in PE epithelium and stroma in patients with severe and mild endometriosis, respectively, as well as MSE epithelium in severe endometriosis samples (Figure 5A-C, Table 5). Positive and negative controls demonstrated specificity of the observed results (Figure 5J-L).

Discussion

General Comments

The main finding of this study is the demonstrated difference in global gene expression in eutopic endometrium from participants with severe versus mild endometriosis, throughout the menstrual cycle. These 2 endometriosis stages are distinct in their clinical presentation, as well as therapeutic and surgical management, although the corresponding scientific literature is limited. The data herein underscore significant molecular and signaling pathway differences between these 2 stages of endometriosis in distinct hormonal milieu, suggesting that eutopic endometrium in severe versus mild endometriosis has different functional capacities.

Comparison of severe versus mild endometriosis samples by cycle phase revealed the dysregulation of several cyclic adenosine monophosphate (cAMP) and/or progesterone regulated gene, such as downregulation of IHH, SST, and TAGLN in ESE and upregulation of DKK1, MAO, IL15, and IL1R1 in MSE. Upregulation of DIO2 and downregulation of TRH transcripts between severe and mild endometriosis samples indicate potential involvement of thyroid hormone homeostasis and metabolism in the pathophysiology of this endometrial disorder.

Women with severe endometriosis experience higher rates of implantation failure during IVF treatment cycles. Of the 25 human receptivity-related genes identified by analysis of

endometrial tissue from healthy fertile women,²⁵ TAGLN and calponin 1 transcripts were dysregulated in MSE from women with severe versus mild endometriosis, suggesting their potential role in the impaired implantation process in women with severe disease.

Dysregulation of Neuregulin Signaling and EGFR in Severe Versus Mild Endometriosis

Of interest is the association of neuregulin signaling with endometriosis. Neuregulin signaling involves ligands for the transmembrane tyrosine kinase receptors ERBB1 (EGFR), ERBB2, ERBB3, and ERBB4—members of the EGFR family.²⁶ Ligand binding activates intracellular signaling cascades and the induction of cellular responses including proliferation, migration, differentiation, and survival or apoptosis in different organs and systems.²⁷ Neuregulin genes, though not regulated themselves in the current study, influence proliferation, migration, and differentiation of epithelial, neuronal, glial, cardiac, and other types of cells.²⁸⁻³⁰ This canonical pathway was highly regulated herein in PE and MSE between severe and mild endometriosis samples. Neuregulin (also known as heregulin) signals through HER3 and HER4 receptors; although no changes were observed herein between disease stages or in different cycle phases.

Epidermal growth factor receptor is the major player of neuregulin-signaling pathway. Epidermal growth factor receptor (ERBB1) expression in normal eutopic endometrium on the mRNA and protein levels during different menstrual cycle phases was demonstrated herein and confirms earlier reports.³¹⁻³³ We have observed that *EGFR* gene expression is increased in eutopic endometrium of women with severe endometriosis compared to women without disease in ESE and MSE, but not PE, and is not regulated in mild endometriosis versus nonendometriosis samples throughout the cycle (Aghajanova et al, unpublished data). Herein, we have found that EGFR is dysregulated in severe versus mild endometriosis throughout the menstrual cycle on both mRNA and protein

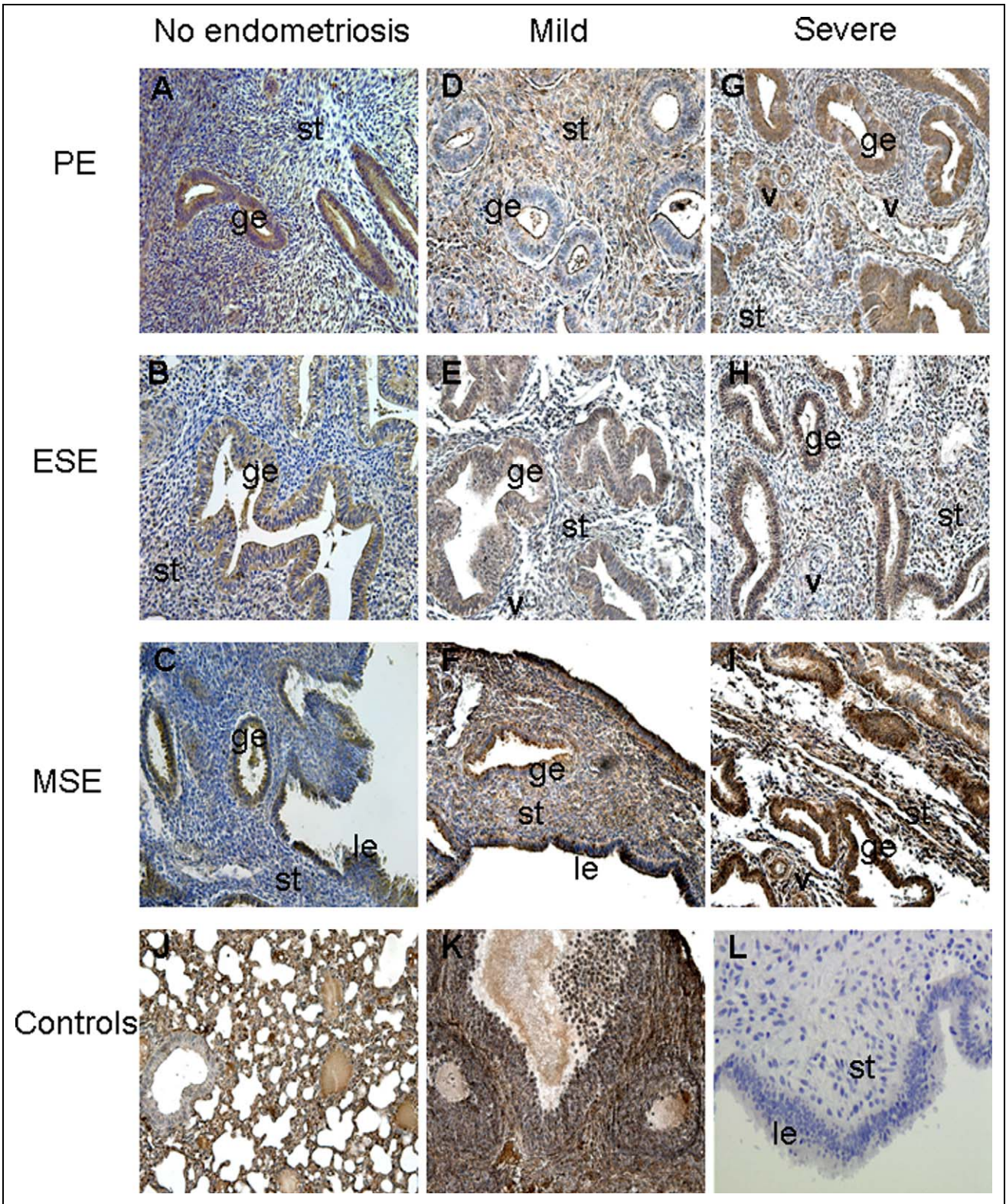


Figure 5. Versican (VCAN) immunoreactivity in endometrial tissue from women without endometriosis (A-C), and women with mild (D-F), and severe (G-I) endometriosis in the proliferative ([PE] n = 4, n = 4, and n = 5, respectively; A, D, G), early secretory ([ESE]; n = 4, n = 3, and n = 7, respectively; B, E, H), and mid-secretory ([MSE]; n = 4, n = 3, and n = 5, respectively; C, F, I) phases of the menstrual cycle. Mouse lung tissue (J) and mouse ovary (K) were used as positive controls. L indicates negative control, nonimmune IgG-treated human endometrium; Le, luminal epithelium; ge, glandular epithelium; st, stroma; v, blood vessel. Magnification $\times 200$.

levels, with the most dramatic difference (upregulation) in ESE. Furthermore, transducer of ERBB2 was upregulated in ESE. Thus, the present study demonstrates differences in EGFR expression between different stages of endometriosis and also supports earlier studies noting involvement of EGF family members in the pathophysiology of endometriosis.^{13,25,34} Interestingly, EGFR is a tumor marker, particularly for epithelial tumors such as colon cancer, lung cancer, prostate cancer, breast cancer, or other solid tumors.^{26,35} Whether it is a marker of a severity of endometriosis remains to be determined.

Dysregulation of ECM Molecules in Severe Versus Mild Endometriosis

This is the first study to demonstrate mRNA expression and immunoreactivity of the ECM proteoglycan VCAN in human endometrium. In participants without endometriosis, VCAN immunoreactivity was weak, especially in PE; whereas, strong immunoreactivity was observed in endometrial stroma from women with severe endometriosis and in epithelium of samples from women with mild disease. Versican can bind to integrins on the cell surface,³⁶ stimulating cell proliferation and inhibiting apoptosis.³⁷ Versican has multiple functions and interactions in different model systems. For example, overexpression of VCAN in a pheochromocytoma cell line upregulates EGFR.³⁸ Also, VCAN expression is increased in endothelial cells with increased migrating capacity.³⁹ Thus, high levels of VCAN may promote an invasive phenotype of endometrial cells in endometriosis by affecting their proliferation, apoptosis, adhesion, and migration, and also may participate in or be causative of the upregulation of EGFR in endometriosis. These functions in endometriosis await further investigation.

Dysregulation of MicroRNAs in Severe Versus Mild Endometriosis

MicroRNA (miRNA) 21 (MIR21) was found to be upregulated on the array, herein, in eutopic endometrium throughout the menstrual cycle in severe versus mild endometriosis. It has recently been shown to be upregulated in eutopic endometrium of women with versus without endometriosis.⁴⁰ Some of the predicted target genes for this miRNA are the tumor-suppressor gene *PTEN* (downregulated 2.18- and 2.3-fold in severe vs mild endometriosis in PE and MSE, respectively), *PDCD4*, *E2F1*, and *TGFBR1*.⁴¹⁻⁴³ Interestingly, downregulation of MIR21 inhibits expression of EGFR in human glioblastoma cells.⁴⁴ Whether there is such a mechanism operating in human endometrial stromal fibroblasts (hESF) remains to be determined.

Herein, we observed the upregulation of DICER1 in ESE and MSE from severe versus mild endometriosis (Supplement Tables 2 and 3). The transcript for DICER1 (*dicer1*, ribonuclease type III), which is a repressor of gene expression due to its involvement in the biogenesis of microRNAs and small interfering RNAs, demonstrates cyclic variation throughout the normal

human menstrual cycle.⁴⁵ Female mice with a conditional knockout of *Dicer1* in mesenchyme-derived cells of the oviducts and uterus are sterile, in part, due to uterine defects.⁴⁶ Although endometrial stromal *Dicer1* expression was absent, the decidualization process was not compromised,⁴⁶ consistent with the recent finding that DICER1 knockdown in hESF does not affect decidualization.⁴⁵ Increased expression of DICER1 in secretory endometrium from women with severe versus mild endometriosis may lead to downregulation of apoptosis-associated genes and dysregulation of adhesion molecules, leading to resistance to apoptosis and increased migratory functions in endometrial cells, as observed with endothelial cells.⁴⁷

Dysregulation of Canonical Pathways in Severe Versus Mild Endometriosis

Several pathways regulated between severe and mild endometriosis are of interest. Severe endometriosis samples exhibited dysregulation of second-messenger signaling pathways, including PI3K/AKT, JAK/STAT, SPK/JNK, and MAPK, confirming recent reports.⁴⁸ Regulation of neurotrophin/TRK (neurotrophic tyrosine kinase) signaling in PE and MSE and axonal signaling in ESE are consistent with the presence of nerve fibers in eutopic endometrium and perhaps their role in the pathogenesis of endometriosis-associated pain.^{49,50} However, other functions of these pathways (and their members) may be operating in endometrium, not related to pain. The current study demonstrates differences in these pathways between severe and mild stages of endometriosis, although pain and stage are not necessarily correlated.^{51,52}

Of note is the involvement of cancer-associated pathways, such as prostate, endometrial, bladder, colorectal, pancreatic cancer, and basal cell carcinoma signaling, suggesting commonalities in the pathophysiology between severe endometriosis and epithelial cancers.

Wnt signaling, NRF2-mediated oxidative stress response signaling (nuclear factor [erythroid-derived 2]-like 2, involved in apoptosis and the oxidative stress response), and retinoid X receptor (RXR) signaling were significantly regulated in secretory endometrium (ESE and MSE; Supplemental Table 2), probably indicating the differences in the endometrial response to progesterone between severe and mild endometriosis.

Summary

Taken together, these data demonstrate the complexity of the processes and gene interactions and pathways involved in the endometrium of women with endometriosis and the molecular differences in the setting of severe versus mild disease. Whether these differences account for the observed differences in clinical presentations of women with severe versus mild endometriosis, that is lower implantation and pregnancy rates in women with severe disease, remain to be determined. The signaling pathways identified may serve for development of targeted therapies to correct the phenotype at the endometrial level.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this article.

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