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Endometrial Gene Expression in Early Pregnancy: Lessons From Human Ectopic Pregnancy

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Abstract

Human endometrium undergoes modifications in preparation for embryonic implantation. This study investigated in vivo the endocrine effects of pregnancy on the endometrium, using the model of ectopic pregnancy. Endometrial biopsies from 9 subjects with ectopic pregnancy (Preg) were compared with 8 and 6 samples of mid and late secretory endometrium, respectively. After hybridizing with Affymetrix HGU133 Plus 2 chips, data were analyzed using GeneSpring GX and Ingenuity Pathways Analysis. From 54 675 genes, 3021 genes were significantly differentiated when mid-secretory endometrium was compared with the Preg (Volcano plot; $P < .05$, ≥ 2 -fold change). The complement and coagulation cascade, phospholipid degradation, glycosphingolipid biosynthesis (globoseries), retinol metabolism, antigen presentation pathway, glycosphingolipid biosynthesis, and O-glycan biosynthesis were main significant canonical pathways found in Preg samples. Validation was done with reverse transcriptase polymerase chain reaction. In conclusion, the ectopic embryo has a significant impact, by an endocrine mechanism, on endometrium, when compared with the window of implantation.

Keywords

Microarray; ectopic pregnancy; transcriptome

Human endometrium is a steroid-responsive tissue that undergoes histologic, structural, biochemical, and programmed gene expression changes throughout the menstrual cycle in preparation for embryonic implantation.^{1–3}

In the very early stages of implantation, embryo attachment to the endometrium and penetration into the endometrial stromal compartment for further growth and development likely occur primarily by paracrine signaling between cells of the conceptus and the decidua.⁴ As gestation proceeds, there are multiple interactions occurring simultaneously. For example, human chorionic gonadotropin (hCG) is secreted from the preimplantation embryo and likely serves paracrine functions at the time of nidation, as well as its known endocrine function of stimulating progesterone production by the corpus luteum for pregnancy maintenance. In addition, there are multiple placental products found in the maternal circulation early in gestation (eg, steroid hormones, placental growth hormone, adrenocorticotrophic hormone [ACTH], human placental lactogen, dimeric inhibin, pregnancy-associated plasma protein-A

[PAPP-A], and pregnancy-specific glycoproteins, among others⁵) that likely are endocrine signals from the conceptus to other tissues in the maternal host, including the endometrium.

We and others have defined the transcriptome of human endometrium, with a focus on the window of receptivity (mid-secretory endometrium, MSE) in nonconception cycles.^{6–9} The endometrial transcriptome, biological processes, and biochemical pathways beyond this time period, in the absence of pregnancy, reflect molecular preparation for tissue desquamation and are uninformative about changes in a conception cycle. In a conception cycle, extending our knowledge into the late secretory phase (week 4 and beyond), has been difficult to investigate because of ethical concerns and confounding contributions of the conceptus/placenta in whole tissue analyses. Thus, alternative approaches have been pursued to unravel trophoblast/endometrium cross-talk, including coculture of human endometrial cells and trophoblast,¹⁰ effects of media from human trophoblasts on decidualized stromal fibroblasts,¹⁰ animal models,¹² and molecular profiling of first trimester abortion specimens.¹³

Ectopic pregnancy, where the conceptus is extrauterine, is a useful model to study the changes in the intrauterine endometrium during pregnancy. In this clinical setting, the endocrine effects of pregnancy on the endometrial transcriptome can be investigated without the confounding effects of placental factors or direct paracrine trophoblast–decidua interactions. Herein, we have compared the endometrial transcriptome from women with tubal gestation to that of endometrium of normal, nonpregnant women during the middle (and late) secretory phase of the menstrual cycle. While acknowledging the limitations of this model, these data have relevance to pregnancy maintenance in normal and abnormal settings and give insight into the endometrial changes that occur inside the uterus during the first trimester of pregnancy without the presence of the embryo inside the uterine cavity. In addition, this study has generated provocative hypotheses for subsequent research.

MATERIALS AND METHODS

Sample Collection and Processing

Endometrial samples from pregnant women ($n = 9$) were obtained from normally cycling subjects undergoing laparoscopic surgery for ectopic pregnancy at Hospital de Clínicas de Porto Alegre, Brazil. After written informed consent, endometrial samples were obtained by suction using a Karman plastic curette (MedGyn Products Inc, Lombard, Illinois), after visual confirmation of the ectopic pregnancy at laparoscopy, but before starting the oviductal surgery. Specimens were snap frozen in liquid nitrogen and stored at -80°C until microarray analysis. Additional samples ($n = 4$) were fixed in 10% formalin and paraffin embedded for microarray validation through immunohistochemistry. These additional samples will permit the generalization of the microarray data. All patients were normo-ovulatory, had not received methotrexate treatment, or other hormonal medications within 3 months of the surgery, and had no or light uterine bleeding. All histology reports from the endometrial biopsy derived from cases of ectopic pregnancy showed the typical decidual changes in endometrial epithelium.

Gene array data from normally cycling women were obtained from the databank of our previous study.¹⁴ The final experiment compared 3 conditions: MSE ($n = 8$); endometrium from subjects with tubal ectopic pregnancy (Preg; $n = 9$), and late secretory endometrium (LSE; $n = 6$). Subject characteristics are shown in Table 1. Samples from LSE were included mainly for ingenuity pathway analysis. This premenstrual phase represents a nonconception cycle, and variations of expression of a certain gene could be seen from the MSE to Preg (conception cycle), or to LSE (nonconception cycle).

Sample Size and Ethical Issues

This study was submitted and approved by the Brazilian National Committee on Ethics in Research (CONEP 1731/2005) and by the Committee on Human Research at University of California, San Francisco (H52883-31577-01). Sample size was calculated according to the literature,¹⁵ considering the median of a standard deviation of 0.2 for HG U133 Plus 2 microarray data set, a minimal 2-fold change for considering a true effect size, a proportion of nondifferentially expressed genes of 0.9, a power of 0.8 and a false discovery rate (FDR) of 0.05. The sample size for these parameters yielded at least 6 samples per group.¹⁵

RNA Preparation/Target Preparation/Array Hybridization and Scanning

Total RNA extraction was performed from each sample using Trizol reagent (Invitrogen, Carlsbad, California) according to the manufacturer's instruction. The RNA preparations were DNase treated and further purified using the RNeasy Mini Kit (QIAGEN, Valencia, CA). The purity was analyzed by both the 260/280 absorbance ratio as well as gel electrophoresis on 1.5% agarose gels. Samples were stored in RNase-free water until further analysis. First-strand and second-strand cDNAs were prepared as described previously.¹⁴ Individual samples were hybridized overnight to high-density human genome (HG) U133 Plus 2.0 Arrays (Affymetrix, Inc, Santa Clara, CA), containing 54 675 gene probe sets. Subsequently, the chips were scanned using an HR3000 scanner, and the data were extracted using the Affymetrix GeneChip Operating Software (GCOS) version 1.1.

Microarray Gene Expression Data Analysis

The intensity values of different probe sets (genes) generated by Affymetrix GCOS were imported into GeneSpring GX version 7.3.1 software (Agilent Technologies, Inc, Santa Clara, CA) for data analysis. The data files (.CEL files) containing the probe level intensities from our previous study,¹⁴ that is, the early proliferative phase, mid and late secretory phase, plus the new data from the endometrium in ectopic pregnancies were preprocessed by Robust Multichip Average (RMA), according to the gene information from the array. The RMA analyses converted the probe-level expression data into gene-level expression data that were normalized, which is the most consistent "good performance."¹⁶ The RMA normalization steps ensured that the distribution of the expression values was comparable across the HG U133 Plus 2 chips. An additional normalization step (per gene: normalize to the median) was used, which ensured that the expression value for one gene across the different conditions was centered on 1, by dividing the expression value by the median value of the expression values for that gene across conditions. This ensured that genes that do not change across conditions got a normalized expression value of 1, allowing for easy visual detection of differentially expressed genes. After identifying sample replicates, gene signals were transformed into a log ratio for normal distribution. Only the data from MSE, LSE, and Preg were used. Quality control was performed filtering the genes below 3 standard errors of the chips. Further filtering was performed to eliminate outlier genes within samples, by using the 95% confidence interval, resulting in the final working gene list consisting of 38 704 genes.

Hierarchical Clustering

The working gene list was submitted to hierarchical clustering to identify samples with similar patterns of gene expression. The smooth correlation for distance measure algorithm (GeneSpring) was used.

Statistical Analysis

The Volcano plot was applied to gene lists from 2 conditions. The values of fold change and *P* values between these 2 conditions were plotted for each gene.¹⁷ The conditions Preg versus MSE were analyzed. The Volcano plot considered genes with equal variance, and genes that

had ≥ 2 -fold change and a P value $< .05$ in the Student's t test were selected. The Benjamini–Hochberg multiple testing correction for FDR at 0.05 was used. A separate analysis, using nonparametric statistics between Preg versus MSE, was performed to confirm the gene list generated by the parametric analysis with Volcano plot. The same settings on fold change and FDR were used. Concordance was identified by the kappa coefficient (κ), which measures pairwise agreement among a set of category judgments, correcting for expected chance agreement. A κ value of $< .20$ indicates weak correlation, $.21$ to $.40$ fair, $.41$ to $.60$ moderate, $.61$ to $.80$ good, and $.81$ to 1 excellent correlation.¹⁸

Further comparisons were made between successive cycle phases using 1-way analysis of variance (ANOVA): MSE, LSE, and Preg with a $P < .05$ for Ingenuity Pathway Analysis (IPA). The Benjamini–Hochberg multiple testing correction for FDR at 0.05 and Tukey as the post hoc test, resulted in a gene list of 32 912 genes that were further filtered on ≥ 2 -fold change, comparing MSE versus Preg and LSE for IPA. Preliminary data obtained from ANOVA were further analyzed through IPA 5.0 (Ingenuity Systems, <http://www.ingenuity.com>) for each individual condition and with comparisons between them.

Canonical pathway analysis: entire data set—Canonical pathway analysis identified pathways from the IPA library of canonical pathways that were most significant to the data set. Genes from the data set that met the 2.0 cutoff of log ratio with a P value $< .05$ and were associated with a canonical pathway in the Ingenuity Pathways Knowledge Base were considered. The significance of the association between the data set and the canonical pathway was measured in 2 ways: (1) A ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed; (2) Fisher's exact test was used to calculate a P value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Pathways graphical representation—IPA Pathways are a graphical representation of the molecular relationships between genes/gene products (http://www.ingenuity.com/company/pdf/Citation_Guidelines_2005-09-13.pdf).

Validation of Microarray Data by Real-Time Polymerase Chain Reaction

First-strand cDNA was generated from 1 μg of total RNA using the iScript cDNA synthesis Kit (Bio-Rad, Hercules, CA). Genes of different expression fold changes in Preg and MSE were selected for validation by real-time polymerase chain reaction (PCR), as described previously,¹⁴ with some modifications. Briefly, real-time PCR was performed using the same samples from Preg ($n = 9$) and MSE ($n = 8$) and were performed in duplicate using the Brilliant SYBR Green PCR kit (Stratagene, La Jolla, CA) according to the manufacturer's specifications. Ribosomal protein L19 (RPL19) was the chosen normalizer.¹⁴ Primers are listed in Table 2. Primer performance and quality were accessed by standard curves with serial dilutions of pooled samples, as described.¹⁴ The efficiencies of amplification (EFF) for each gene were obtained from Mx3005P software (Stratagene, La Jolla, CA) in the exponential phase of the amplification curve. PCR thermal profiles were run using the Mx3005P (Stratagene).¹⁴ Primers with standard curves that showed 100% amplification efficiency ($\pm 10\%$) were used in subsequent sample analyses. Ct values were calculated by the Mx3005P software based on fluorescence intensity values after normalization with an internal reference dye and baseline correction.¹⁴ Statistical analysis of the RT-PCR data were performed using the Mann–Whitney test of the relative expression of each group, with $P < .05$.

Immunohistochemistry

Additional formalin-fixed paraffin-embedded endometrial samples (n = 4 Preg and n = 4 MSE) were used for immunostaining the highest gene expressed in our microarray analysis (matrix metalloproteinase-7, MMP7). Archived breast cancer samples were used as positive controls. Negative control was obtained by using low concentration (2 µg/mL) of primary antibody. Sections were cut (8 µm), deparaffinized, hydrated and treated with 0.3% H₂O₂–methanol for 30 minutes at 25°C to reduce endogenous peroxidase activity. Nonspecific sites were blocked with 2% normal goat serum. After 3 × 5 minutes phosphate buffered saline (PBS) washings, mouse monoclonal anti-MMP7 active form (IM47, 15 µg/mL; Calbiochem, San Diego, CA) was incubated overnight at 4°C in a humidity chamber. After additional 3 × 5 PBS washings, another blocking with 2% normal goat serum followed by 1 hour of incubation with biotinylated anti-mouse IgG (BA-9200, Vector Laboratories, Burlingame, CA) at 25°C was performed. Slides were incubated with avidin DH-biotinylated horseradish peroxidase H complex (Vectastain Standard ABC kit, Vector Laboratories) for 1 hour at 25°C followed by immersion in 3,3'-diaminobenzidine tetrahydrochloride (Aldrich Chemical Co, St Louis, Missouri) at 150 mg/200 mL 0.05 M Tris buffer containing 0.002% H₂O₂ for 10 minutes with constant stirring. Sections were exposed to osmium vapors and counterstained with 0.05% toluidine blue in 30% ethanol, dehydrated, cleared in xylene and mounted with Permount (Fisher Scientific, Hanover Park, IL). Photographs were taken using a SPOT-4 Megapixel Digital Color Camera System (Diagnostic Instruments, Inc, Sterling Heights, Michigan) attached to a Nikon ECLIPSE E600 microscope and prepared using SPOT image processing software.

RESULTS

Gene Expression Data Distribution and Hierarchical Clustering

The gene expression data were normally distributed with log ratio transformation in all 3 physiologic groups. The concordance in the gene list generated between parametric and nonparametric analyses was excellent, with the κ value at 0.95. Figure 1 shows the dendrogram of sample clustering and the heatmap of gene expression for all 3 physiologic groups (MSE, LSE, and Preg). The hierarchical tree with branches indicates the relationships between the different samples, using Pearson correlation for similarity measures, and demonstrates that sample groups segregate into 3 main branches, showing similarity within the samples within each physiologic state but clear differences between sample groups.

Volcano Plot/ANOVA

When Preg was compared with MSE, the parametric Volcano plot/ANOVA analysis generated a gene list of 3021 genes, of which 1990 were upregulated and 1031 were downregulated. Analysis of LSE versus MSE yielded a list of 973 genes, of which 515 were upregulated and 458 were downregulated. The genes that are upregulated and downregulated ≥ 4 -fold are in listed in Tables 3 and 4, respectively.

Ingenuity Pathways Analysis

ANOVA analysis comparing the MSE, LSE, and Preg groups and considering only a $P < .05$, without considering a fold change, resulted in preliminary composite list of 32 912 genes for uploading to IPA, which can show the changes of the genes over these 3 conditions. These genes with their corresponding gene identifiers, log ratio expression values, and P values were uploaded into the application, and after using a 2-fold cutoff, the analysis identified 774 genes eligible for generating networks.

Canonical pathways analysis—From all canonical pathways from the IPA, 7 pathways reached the 2-fold cutoff in the Preg group. Natural killer cell signaling pathway had a significant increase in LSE, but no expression in Preg (Figure 2). From these pathways, the complement system has the highest significant expression (Figure 2). In the early stages of pregnancy, although factor B is upregulated, the alternate pathway is inhibited by complement factor H (CFH), which has a 4-fold increase ($P < .0001$). There is no expression of activated C3. The classical pathway is not active either because C4b is not expressed or regulated. The membrane attack complex, composed of C5–C9 (in the late steps of complement activation), did not reach the 2-fold preestablished cutoff (Figure 3). The major histocompatibility complex, class I, E in the antigen presentation pathway (Figure 4) was upregulated in the Preg samples.

Validation of Microarray Data by Real-Time PCR

Real-time PCR analysis confirmed the microarray data of regulation of a subset of 21 genes (except CFI, which was only identified in IPA analysis by statistical difference, not by fold change). Statistical analyses for each validated gene using all samples ($n = 19$) are listed for comparison in Table 5.

Validation of Microarray Data by Immunohistochemistry

The immunostaining for active form of MMP7 was present in the stromal and glandular cells of Preg samples, while in MSE, weak or absent expression was found in stromal cells (Figure 5). Positive and negative controls stained positively and negatively, respectively (data not shown).

COMMENT

The endometrial changes that occur between the mid-secretory phase of the menstrual cycle until the pregnancy have been difficult to extrapolate given the ethical and scientific limitations involved in studying early pregnancy. Several array data sets compiled from human pregnancy are available, but they used in vitro models,^{10,11} or in vivo models in the presence of the trophoblast.¹³ By analyzing the endometrium from the uterine cavity in the setting of a tubal pregnancy, we were able to identify dramatic changes that reflect the endocrine milieu of pregnancy without the contribution of the conceptus inside the uterine cavity and this may be the reason why many differences were found comparing our model to the published data. For instance, Chen et al¹³ used a human in vivo model comparing the decidua gene profile from an intrauterine pregnancy to the transcriptome of the trophoblast, and they presented a list of 48 genes that were upregulated in the decidua. From these genes, only 7 were in common with our gene list (IGFBP1; thrombin receptor-like 2; protein tyrosine phosphatase, receptor type; AXL receptor tyrosine kinase; CD54; NADH dehydrogenase [ubiquinone] 1; and microsomal glutathione S-transferase 1). Furthermore, the highly expressed presence of insulin receptor found by Chen et al¹³ and Popovici et al,¹¹ who used a coculture model, was not found in our study, underlying the difference made by the local presence of the trophoblast. The comparison between the transcriptome of MSE to the decidua in a setting of an ectopic pregnancy gives valuable insight into the modifications of genes, gene families, and pathways that occur from the window of implantation (MSE) to the early gestation period as a result of secretion of products from the ectopic trophoblast. We are not able to distinguish if the differences found are derived from the products of the corpus luteum or from the ectopic trophoblast and embryo. A study having women taking estradiol and progesterone for 6 weeks could help explain it, but it could face ethical concerns. The numerous genes, gene families, and canonical pathways that are upregulated in the decidua in the setting of tubal gestation, compared with MSE, are subject to several interpretations. Some of the genes and pathways may be regulated in an endocrine fashion by products from the corpus luteum and the ectopic placenta, or from a prolonged exposure of their products. Caution regarding interpretation of these results is

required given that endocrine parameters in ectopic pregnancy may vary from that of normal pregnancies. Some may be absent or have limited expression in endometrial decidua with ectopic gestation because they ordinarily result from paracrine interactions between the trophoblast and the decidual cells in a normal pregnancy. There may be a threshold value at which gene expression in the decidua is maintained or even stimulated, and below which, gene expression may be suppressed. Also, the presence of a placenta in the fallopian tube may result in paracrine signaling between the trophoblast and oviductal cells that could result in secreted products (eg, cytokines, chemokines) affecting the uterine decidual transcriptome. Of note, the majority of our LSE samples had leiomyomas, which could be a limitation of our study. Because our main analysis was based on MSE versus Preg, the potential impact of this is minimal based on our final analysis.

In this study, we compared gene expression profiles and pathways of intrauterine decidua in the presence of ectopic pregnancy with 6.5 ± 1.04 (mean $\pm$ SEM) weeks with MSE, which is the third week of the menstrual cycle (peak of progesterone) in the nonpregnant state. It has been reported that between 5 and 9 weeks of gestation, circulating progesterone and hCG levels are lower in ectopic pregnancy, compared with normal intrauterine pregnancy.¹⁹ Mean values of progesterone and β -hCG in the latter were 21.3 ± 6.8 ng/mL and $37\,533 \pm 30\,133$ mUI/mL, compared with ectopic pregnancy values of 6.3 ± 3.1 ng/mL and 4106 ± 1762 mUI/mL, respectively. However, in our samples, we found mean hCG levels of $16\,665 \pm 26\,267$ mUI/mL from the available data ($n = 8$). Mean progesterone in the mid-luteal phase of a nonconception cycle is 10.83 ng/mL.²⁰ Although we did not measure progesterone levels in our subjects, which precludes an objective conclusion about the role of progesterone over the endometrium in this setting, the overall gene profiles and regulated pathways observed herein are not consistent with global progesterone withdrawal at the level of the endometrium (as in LSE in nonconception cycles^{14,21}) or with an inadequate progesterone response. In support of this is the observation (Table 3) that IGFBP-1, IGFBP-6, somatostatin, HTR2B, HLA-DQB1, RGS2, HBB, IL1R type II, and ADAMTS2 are all upregulated in Preg decidua versus MSE, and are known to be upregulated in either endometrial stromal fibroblasts in response to progesterone²² or in MSE in nonconception cycles.^{14,23} Another possibility for these findings is a prolonged progesterone exposure.

A study that most resembles our model was used by Sherwin et al.¹² They used intrauterine infusion of hCG in the baboon, and found regulation of numerous genes in endometrium that are important in embryo attachment, endometrial remodeling, antioxidant defense, and modulation of the immune response. Our 6-week decidua data demonstrate that 30 genes out of the 61 genes regulated by hCG observed by Sherwin et al.¹² are in common. Of note, MMP7, members of the complement and coagulation cascades, and interleukin-8 (IL-8) are among these (Table 6). The similarities are even greater if the pathways involved are considered, such as the complement cascade and the Wnt pathway (see below). Yet, IL-6 levels reported by Sherwin et al.¹² were not found in our study. IL-6 was significantly upregulated in MSE, compared with Preg samples, by RT-PCR analysis (Table 5), for unclear reasons. Furthermore, our results are in accordance to those found by Tabibzadeh et al.,²⁴ in that IL-6 mRNA expression increases progressively across the menstrual cycle (in our microarray data: MSE: 1.122, LSE:1.625, Preg:1.879), although they were not statistically significant ($P = .3$).

The different genes across the 3 different conditions (MSE, LSE, and Preg) were used for IPA analysis, where the variations of the genes across the conditions can be seen. We decided to validate the main genes identified in the microarray analysis that were relevant for endometrial receptivity or were derived from IPA analysis, such as ETS-1, which could explain the upregulation of MMP7. Of note, MMP7 is one of the products of the Wnt/ β -catenin pathway, and this pathway is probably downregulated, and Sherwin et al.¹² reported the expression of this MMP. Because of these, we decided to validate MMP7 by IHC. Genes with a wide function,

such as cytochrome P450, were precluded from the validation. However, we focus our discussion on the main processes between MSE and Preg samples because they represent the physiological timeline from uterine receptivity through early pregnancy.

DECIDUALIZATION/APOPTOSIS AND PROLIFERATION PREVENTION

Inhibin β A/Somatostatin (SST)/Forkhead Box O1 (FOXO1A)/IGFBP1/TIMP3

Inhibin β A is upregulated in early pregnancy, and this finding is corroborated by Otani et al,²⁵ who reported intense immunoreactivity in decidual stromal cells during early pregnancy. Inhibin β A promotes decidualization per se.²⁶ Progesterone (and activation of the protein kinase A pathway) is a regulator of FOXO1A, prolactin, IGFBP1, TIMP3, SST, endometrial bleeding-associated factor (EBAF), and BIM (bcl-2 interacting mediator of cell death) mRNAs in endometrial stromal fibroblasts^{27,28} in vitro and in vivo studies, and all are markedly induced during decidualization process.¹⁴ Herein, mRNA levels of FOXO1A, IGFBP1, TIMP3, and SST were all upregulated in our microarray data, and they were validated by RT-PCR analyses, compared with MSE, except for TIMP3 that did not reach statistical significance. BIM mRNA levels were upregulated in microarray analysis as well. Interestingly, placental corticotrophin releasing hormone (pCRH), whose plasma levels progressively increase during pregnancy,²⁹ increases FOXO1A and IGFBP1 mRNA expression through signaling via the glucocorticoid receptor,³⁰ which is upregulated in our microarray analysis (1.4-fold change; $P = .03$), suggesting that the decidua may be a target for placental CRH in an endocrine fashion. Labied et al²⁸ have shown that withdrawal of progesterone from human decidualized endometrial stromal cells elicited reaccumulation of FOXO1A in the nucleus and apoptosis, whereas in the presence of progesterone, FOXO1A is accumulated in the cytoplasm of the cells and apoptosis is precluded. Thus, these data are consistent with our findings, and we may infer that progesterone levels found in early pregnancy may preclude endometrial cellular apoptosis through FOXO1A. Furthermore, endometrial proliferation is precluded by inhibition of the Wnt/ β -catenin pathway, through the upregulation of DKK-3 and SFRP1, as has been shown by Tulac et al³¹ in an in vitro study.

IMMUNOLOGICAL PROCESSES

Immune interactions at the maternal–fetal interface play an important role in implantation biology. Some participants have been found to be regulated in Preg versus MSE, including members of the complement cascade, such as complement factor H (CFH), and from the antigen presentation pathway (HLA-E). The complement inhibition is an absolute requirement for normal pregnancy, where Crry deficiency in utero leads to progressive embryonic lethality by activation of C3 fragments and polymorphonuclear leukocytes.³² In humans, during the window of implantation, maximal DAF expression, at the mRNA and protein levels, occurs by day 22 of the menstrual cycle and is sustained thereafter in whole tissue and in endometrial luminal and glandular epithelium.³³ Nevertheless, our results showed that only the alternative complement cascade is inhibited by CFH, in decidua in the presence of ectopic pregnancy, as demonstrated by microarray and RT-PCR analysis. The alternative pathway is related to autoantibodies and the initiation of the inflammatory cascade.³⁴ Surprisingly, the lectin and the classical pathways are not inhibited. These pathways are activated by bacterial surface and antigen–antibody complex, respectively,³⁵ showing that the decidua is prepared to defend against bacterial infections. Similarly, another antibacterial mechanism, through lipolcain-2 (LCN2) was found in our microarray and RT-PCR analysis. LCN2 modulates inflammation/infection, and prevents growth of microorganisms by depriving them of iron.³⁶ Furthermore, LCN2 is upregulated by trophoblast products, such as glucocorticoid, retinoic acid,³⁷ IGF-I,³⁸ E₂,³⁹ and progesterone,⁴⁰ and downregulated by activation of the Wnt pathway.⁴¹ All these data are in accordance with the current findings. The physiologic importance of LCN2 in pregnancy can be derived from the genotype of LCN2 $-/-$ knockout mice.⁴² LCN2 $-/-$ females

have a normal phenotype but are less fertile than wild-type mice. In wild-type mice, LCN2 mRNA and protein in the decidua remain high immediately after fertilization and early pregnancy (on days 1 and 2 of a 20-day gestation), and decline with progression of pregnancy.⁴³ These findings are comparable to ours in early pregnancy.

It is well established that uterine natural killer (NK) cells are particularly abundant around the time of implantation and during early pregnancy.⁴⁴ However, the IPA analysis showed a significant reduction of NK cells signaling in Preg samples, compared with LSE as shown in Figure 2. A possible explanation for this finding can be related to increased expression of HLA-E, a nonclassical major histocompatibility complex (MHC) class I molecule.⁴⁵ HLA-E has been related to the inhibition of the killing activity of NK cells.⁴⁶ HLA-E complex is elevated in Preg samples, and the presence of HLA-E in decidua may protect an incoming embryo from NK cell attack. Indeed, there is growing evidence showing that decidual NK cells control trophoblast invasion, and this control occurs through the release of an interferon-inducible protein-10 (IP-10), and IL-8.⁴⁷ In our analysis, IL-8 mRNA is significantly upregulated in Preg samples, compared with the MSE, which is consistent with the results found by Critchley et al.²¹ These authors demonstrated IL-8 immunostaining primarily in perivascular sites in decidua derived from ectopic pregnancy.²¹ Upregulation of IL-8 has been reported at the site of endometrial and embryo interactions in most,^{10–12} but not all¹³ studies, and hCG upregulates its expression.¹² IL-8 has a potent chemotactic activity on neutrophils migration,⁴⁸ and this migration involves the expression of calgranulin A/B complex (S100A8/A9).⁴⁹ This complex is upregulated in our microarray analysis, and S100A8 was confirmed by RT-PCR.

EXTRACELLULAR REMODELING AND ANGIOGENESIS

MMP7 and MMP11 are the most highly regulated genes in the Preg samples (Table 3). They are known to be downregulated in endometrial epithelium and stromal fibroblasts, respectively, in response to progesterone, although upregulation of MMP7 in response to hCG was demonstrated in the baboon model described above.¹² MMPs are regulated by transforming growth factor beta (TGF β) family members, and are activated by other proteases and inhibited by tissue inhibitor of metalloproteases (TIMPs).⁵⁰ Immunohistochemical staining of endometrial decidua derived in the setting of tubal pregnancy confirmed the expression of the active form of this collagenase (Figure 5). Interestingly, it is one of the products of the Wnt/ β -catenin signaling pathway activation.⁵¹ However, because DKK-3 and SFRP1, inhibitors of the Wnt/ β -catenin pathway, are upregulated in the decidua of ectopic pregnancy (Table 5), the marked upregulation of MMP7 is likely controlled by other mechanisms. Therefore we decided to validate the active form of the protein by IHC. Candidates include v-ets erythroblastosis virus E26 oncogene homolog 1 (avian) (ETS-1), which was confirmed by RT-PCR analysis, and retinoic acid, which is highly expressed in Preg samples (Figure 2). Both ETS-1 and retinoic acid are known stimulators of MMP7,⁵² as well hCG.¹² With regard to MMP11, Popovici et al¹¹ did not find regulation of MMP11 in a coculture model of trophoblasts and endometrial stromal cells, and further studies are needed to understand the regulation of this MMP in decidua.

Periostin was highly upregulated in Preg samples by microarray analysis (7.4-fold) and confirmed by RT-PCR (14.11-fold). There are no published data on POSTN in the human endometrium, although this secreted and soluble extracellular matrix protein⁵³ has been identified in a wide range of normal adult tissues,^{54–57} and cancers.^{58,59} Its function is related to extracellular matrix remodeling,⁶⁰ enhancement of motility and invasiveness of cancer cells, and is a ligand for the integrins $\alpha\beta$ 3 and $\alpha\beta$ 5.⁵⁴ It may serve similar functions for trophoblasts in decidua. TGF β 1 increases the expression of periostin,⁵⁵ and TIMP-2 and periostin are codistributed on extracellular matrix.⁶¹ Herein, TGF β 1 was found to be upregulated 2.27-fold

($P = .0007$) and TIMP2 2.67-fold ($P = .007$) in Preg samples, consistent with a role and distribution of POSTN in other systems.

Along with its coagulation activity, PAI-1 exerts control over trophoblast invasion.⁶² In our study, PAI-1 was upregulated in Preg samples. Lockwood et al⁶³ demonstrated that PAI-1 protein and mRNA expression is enhanced by estradiol and medroxyprogesterone acetate in decidualized stromal cells from luteal phase and from first trimester endometrium cultured in type 1 collagen gels. PAI-1 is important for pregnancy maintenance, as women with homozygosity for PAI-1 polymorphism have a higher relative risk of early pregnancy loss.⁶⁴ The upregulation of PAI-1 without the local presence of the trophoblast is a new finding and needs further investigation.

Thus, MMPs play a key role in angiogenesis by degrading the extracellular matrix and permitting the migration and tube formation of endothelial cells,⁶⁵ and IL-8 induces angiogenesis.⁶⁶ Lumican is involved in extracellular matrix remodeling,⁶⁷ as are MMP7, MMP11, periostin, and IL-8. These observations underscore the importance of extracellular matrix remodeling in the decidua in preparation of the highly invasive trophoblast.

In conclusion, we have demonstrated that the transcriptome of the decidua in the setting of an ectopic pregnancy is likely influenced by trophoblast products in an endocrine fashion, compared with nonpregnant, secretory endometrium. The validated genes in this study are related to the coagulation cascade and trophoblast invasion (PAI-1), immunological processes (HLA-E, IL-8, S100A8, CFH), endometrial tissue remodeling (MMP7, 11, periostin, lumican, IL-8), decidualization, and apoptosis/proliferation prevention (FOXO1A, IGFBP-1, SST, DKK3, SFRP1). Our findings are in accordance with studies by others that used in vitro human and animal models, suggesting that although paracrine interactions are important in the invasive phase of implantation, endocrine effects are also likely important, as well. We also have found genes that have not been described before in the endometrium of early human pregnancy, such as complement factor H, periostin, lipocalin 2, and lumican, as well as CFI in MSE, opening new fields for further investigation. Importantly, in our list of upregulated genes in Preg versus MSE, we did not observe upregulation of GRO-1, DKK-1, and TIMP-3, previously observed in cultured cells of human endometrial stromal fibroblast in the presence of trophoblast,¹³ and in stromal fibroblasts treated with trophoblast culture medium.¹⁰ These observations strongly support either paracrine regulation of these factors or a threshold of response to progesterone with regard to specific gene expression. This is in contrast to IL-8, FOXO1A, SST, and IGFBP1, which appear to be regulated by endocrine factors, such as progesterone, hCG, and other placental products that prepare the endometrium for implantation.

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Figure 1.

Hierarchical clustering. The heat map represents the relative expression levels of genes in each individual sample. Each horizontal line represents a single gene and each column represents a single sample. Samples cluster according to the condition (MSE, mid-secretory endometrium; LSE, late secretory endometrium; Preg, tubal ectopic pregnancy).

Figure 2.

Canonical pathways. Ingenuity Pathways Analysis identified the most significant genes from the data set. The bars represent the canonical pathway, which is associated with the genes uploaded from the gene list generated from GeneSpring that met the 2.0 cutoff of log ratio with a P value $< .05$. Of note, in natural killer cell signaling pathway, the high expression of this pathway in LSE is suppressed in Preg, showing that Preg condition has a significant impact over this particular pathway. MSE indicates mid-secretory endometrium; LSE, late secretory endometrium; Preg, tubal ectopic pregnancy.

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Figure 3.

The complement cascade. This figure represents the complement cascade with 3 pathways of the complement cascade represented. The lectin and classical pathways are antigen–antibody complex related, whereas the alternative is continuously activated in blood plasma. The alternative cascade leads to the destruction of host cells. During the mid-secretory phase, DAF is upregulated and inhibits all pathways of the complement cascade. However, in early pregnancy, only the alternative pathway is inhibited by CFH. The intensity of the node color indicates the degree of significant upregulation (red), downregulation (blue) with a cutoff >2-fold; gray nodes represent genes present in the data set, but which did not reach the cutoff; white nodes are genes that belong to the canonical pathway but are not present in the data set. Nodes with double circle have more than one member. The bar graph near the node represents the gene expression in MSE, LSE, and Preg samples, respectively, from left to right. Numbers below nodes represent fold change between Preg and MSE (upper lane), and *P* value (bottom lane). C indicates complement component; M, mannose-binding lectin (protein C) 2, soluble (opsonic defect; SERPING1, serpin peptidase inhibitor, clade G (C1 inhibitor), member 1 (angioedema, hereditary); MASP, manna-binding lectin serine peptidase; BF, complement factor B; DAF, decay accelerating factor for complement; MCP, CD46 molecule, complement

regulatory protein; CR1, complement component (3b/4b) receptor 1; DF, complement factor D (adipsin); IF, complement factor I; HF1, complement factor H; MSE, mid-secretory endometrium; LSE, late secretory endometrium; Preg, tubal ectopic pregnancy.
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Figure 4.

Representation of the antigen presentation pathway. The expression of the HLA-E is higher in Preg, whereas it is downregulated in MSE and LSE. HLA-E inhibits the activation of the natural killer (NK) cells, thus modulating the immune system. The intensity of the node color indicates the degree of significant upregulation (red), downregulation (blue) with a cutoff >2 -fold; gray nodes represent genes present in the data set, but which did not reach the cutoff; white nodes are genes that belong to the canonical pathway but are not present in the data set. Nodes with a double circle have more than one member. The bar graph near the node represents the gene expression in MSE, LSE, and Preg samples, respectively, from left to right. Numbers below nodes represent fold change between Preg and MSE (upper lane), and P value (bottom lane). HLA indicates major histocompatibility complex, class I; MP1, major histocompatibility complex, class I-related; MHC I- β , beta-2-microglobulin; MHC I- α , major histocompatibility complex; LMP2, proteasome (prosome, macropain) subunit, beta type, 9 (large multifunctional peptidase 2); LMPX, proteasoma (prosome, macropain) subunit, beta type, 5; LMPY, proteasoma (prosome, macropain) subunit, beta type, 6; LMP7, proteasome (prosome, macropain) subunit, beta type, 8 (large multifunctional peptidase 7); TAP1, transporter 1, ATP-binding cassette, subfamily B (MDR/TAP); TAP2, transporter 2, ATP-binding cassette, subfamily B (MDR/TAP); TPN, TAP binding protein (tapasin); CNX, calnexin; CALR, calreticulin; CLIP, CD74 molecule, major histocompatibility complex, class II invariant chain; MSE, mid-secretory endometrium; LSE, late secretory endometrium; Preg, tubal ectopic pregnancy.

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Figure 5. Immunostaining for active form of MMP7 in mid-secretory endometrium (MSE) (A) and Preg (B). Note the immunostaining of decidualized endometrium cells in the stroma (arrow) in the presence of ectopic pregnancy (B), whereas no staining is observed in the stroma of MSE (black arrow), only in the glands (arrowheads).

Characteristics of the Samples

Table 1

Patient ID	Cycle Phase	Age (Years)	Indication/Diagnosis	hCG mIU/mL	Ethnicity	Medications	Total
610	MSE	49	Leiomyomata	–	Caucasian		8 ^a
617		42	Pelvic pain (no endo)	–	Caucasian		
626		42	Uterine prolapse	–	Caucasian	Allegra, Advil	
M153		34	Endometrial biopsy-volunteer	–	Caucasian		
659		46	Uterine prolapse	–	Caucasian		
G98A		30	Endometrial biopsy-volunteer	–	Caucasian		
M158		23	Endometrial biopsy-volunteer	–	Black		
M163		33	Endometrial biopsy-volunteer	–	Caucasian		
442	LSE	43	Leiomyomata; adenomyosis	–	Black	Astelin, Flonase	6 ^a
449		39	Leiomyomata	–	Caucasian		
462		39	Leiomyomata	–	Asian		
614		43	Uterine prolapse	–	Other	Detrol LA	
576		41	Ovarian cyst, endometrial biopsy	–	Unknown		
619		44	Leiomyomata	–	Caucasian		
1	Preg	24	Ectopic pregnancy, 7.6 weeks	n/a	Caucasian		13
2		36	Ectopic pregnancy, 6 weeks	5779	Caucasian		
3		38	Ectopic pregnancy, 6 weeks	10 910	Caucasian		
4		31	Ectopic pregnancy, 6 weeks	n/a	Black		
5		36	Ectopic pregnancy, 7 weeks	n/a	Black		
6		25	Ectopic pregnancy, 6 weeks	762	Caucasian		
7		41	Ectopic pregnancy, 5 weeks	29 950	Caucasian		
8		24	Ectopic pregnancy, 6.5 weeks	2350	Caucasian		
9		43	Ectopic pregnancy, 8.5 weeks	77 293	Black		
10		20	Ectopic pregnancy, 6 weeks	2096	Caucasian		<i>b</i>
11		31	Ectopic pregnancy, 7 weeks	4176	Black		<i>b</i>
12		25	Ectopic pregnancy, 1 trimester	n/a	Caucasian		<i>b</i>
13		28	Ectopic pregnancy, 8.5 weeks	n/a	Caucasian		<i>b</i>

Abbreviations: MSE, mid-secretory endometrium; LSE, late-secretory endometrium; Preg, tubal ectopic pregnancy; –, not applicable; n/a, not available.

^aFrom reference 14.

^bSample used for validation but not for array analysis.

Table 2

Primers Used for Microarray Gene Expression Validation

Gene	Forward Primers 5'-3'	Reverse Primers 5'-3'	Amplicon Length (bp)	Annealing Temperature (°C)
Dickkopf homolog 3 (DKK3)	TCACATCTCTGGGAGACGA	GGGAGGTAAGTTTGGCCAGGT	101	56
Forkhead box O1A (FOXO1A)	AAGAGCGTGCCCTACTTCAA	CTGTTGTGTCCATGGATGC	114	58
Inhibin, beta A (INHBA)	AGAGGTGGCTCAAAGTGT	GGATGGTGACTTTGGTCCTG	157	60
Insulin-like growth factor binding protein 1 (IGFBP1)	CTATGATGGCTCGAAGGCTC	TTCCTTGTGCAGTTTGGCAG	156	56
Interleukin 8 (IL-8)	GAAGAGAGCTCTGTCTGGAC	TCACTGGCATCTTCACTGAT	131	56
Interleukin 6 (IL-6)	AGTGAGGAACAAGCCAGAGC	CAGGGGTGGTTATTGCATCT	234	54
Lipocalin 2 (LCN2)	GAGTTACCTCGTCCGAGTGG	CGAAGTCAGCTCCTTGGTTC	127	58
Matrix metalloproteinase 11 (MMP11)	CTTCCGAGGCAAGGACTACT	AGAAATCAGGACCCACGAGA	227	58
Matrix metalloproteinase 7 (MMP7)	TTGGGTATGGGACATTCCTC	TGAATGGATGTTCTTGCCTGA	176	56
Periostin (POSTN)	CAAAATGTCTGTGCCCTTCAA	CAGCCTTTCAITTCCTTCCAT	164	56
Preprosomatostatin (somatostatin)	CCCAGACTCCGTCAGTTTCT	ATCAATCTCCGTCTGGTTGG	114	58
Ribosomal protein L19 (RPL19)	GCAGATAATGGGAGGAGCC	GCCCATCTTTGATGAGCTTC	207	58
S100 calcium binding protein A8 (S100A8)	CAGCTGTCTTTTCAGAAGACCTG	TGAGGACACTCGGTCTCTAGC	153	58
Secreted frizzled-related protein 1 (SFRP1)	TGTTGTCTCTCCCTGTGACAA	CCAACTTCAGGGGCTTCTTC	168	58
PAL-1	ACCAAGAGCCCTCCACGTC	TCCAGTTTGTCCCAGATGA	185	56
V-ets erythroblastosis virus E26 oncogene (ETS-1)	CCAATCCAGCTATGGCAGTT	CCACGGCTCAGTTTCTCTATA	180	56
Complement factor H (CFH)	TGCTGTATGCACCTGAATCTGG	GGGTTGCAGGATAAAACCA	164	56
Complement factor I (CFI)	CTGGATACACCCCGACCTTA	CGATTTCCGTAAAACTTAGAGC	180	56
Major histocompatibility complex, class I, E (HLA-E)	GAGGAGCTACTCTAAGGCTGA	CGTGAGGAAATCCTGCATCT	104	56
Nuclear antigen Sp100 isoform 2	CAGAAACCAACCAAGCACAAAGA	GCAAAAAATGGTCTTCCATGTC	188	56
Decay accelerating factor (DAF)	TGGAAACCACTTCAGGTACTA	TCTCCTTGCTCTGTTGACAT	260	58
Tissue inhibitor of matrix metalloproteinase 3 (TIMP3)	TGAACTATCGGTATCACCTG	CATCTCGGTACCAGCTGCA	190	56

Table 3**Genes Upregulated Between Preg and MSE**

Gene Name	Entrez Gene ID	Fold Change	P Value
MMP7 (matrix metalloproteinase 7 [matrilysin, uterine])	4316	10.99	.0004
MMP11 (matrix metalloproteinase 11 [stromelysin 3])	4320	10.04	.00145
LCN2 (lipocalin 2)	3934	8.946	.000268
S100A8 (S100 calcium binding protein A8)	6279	8.913	.001
SST (somatostatin)	6750	8.793	.012
INHBA (inhibin, beta)	3624	8.068	.00745
POSTN (periostin, osteoblast specific factor)	10631	7.499	.000115
WISP1 (WNT1 inducible signaling pathway protein 1)	8840	7.197	.000208
DKFZP586H2123 (regeneration associated muscle protease)	25891	6.733	1.94E-05
CYP24A1 (cytochrome P450, family 24, subfamily A, polypeptide 1)	1591	6.34	.00185
CFH; CFHL1 (complement factor H)	3075; 3078	6.204	4.97E-05
PDZK1IP1 (epithelial protein upregulated in carcinoma, membrane associated protein 17)	10158	6.19	.00307
ZD52F10 (dermokine)	93099	6.009	5.99E-07
IGFBP1 (insulin-like growth factor binding protein 1)	3484	5.93	.0316
IGH@; IGHG1; IGHG2; IGHG3; IGHM (immunoglobulin heavy chain)	283650; 3493; 3494	5.872	.00151
IGHA1; IGHA2; MGC27165 (immunoglobulin heavy constant alpha 1)	3492; 3500; 3501; 3502; 3507	5.872	.000569
GEM (GTP binding protein overexpressed in skeletal muscle)	2669	5.808	.00257
URB (steroid-sensitive protein 1)	151887	5.668	3.21E-06
KYNU (kynureninase [L-kynurenine hydrolase])	8942	5.652	7.89E-05
C1orf168 (chromosome 1 open reading frame 168)	199920	5.609	4.99E-06
IGFBP6 (insulin-like growth factor binding protein 6)	3489	5.51	.000833
MFAP4 (microfibrillar-associated protein 4)	4239	5.468	.00252
ITGB6 (integrin, beta 6)	3694	5.455	.00052
TACSTD2 (tumor-associated calcium signal transducer 2)	4070	5.396	3.93E-06
IL8 (interleukin 8)	3576	5.314	.0138
ITGA9 (integrin α 9)	3680	5.195	6.56E-05
HTR2B (5-hydroxytryptamine [serotonin] receptor 2B)	3357	5.122	.000852
HLA-DQB1 (major histocompatibility complex, class II, DQ beta 1)	3119	5.093	4.09E-05
LTBP2 (latent transforming growth factor beta binding protein 2)	4053	5.03	.000111
IGL@; IGLC1; IGLC2; IGLV3-25; IGLV2-14; IGLJ3 (immunoglobulin lambda locus; immunoglobulin lambda constant 1)	28793; 28815; 28831; 3535; 3537; 3538	4.982	.00238
RGS2 (regulator of G-protein signaling 2)	5997	4.977	.000167
PTGS2 (prostaglandin-endoperoxide synthase 2)	5743	4.974	.00521
FXYP2 (FXYP domain containing ion transport regulator 2)	486	4.952	.0381
HBB (hemoglobin, beta)	3043	4.913	.0075
LOC200169 (hypothetical protein LOC200169)	3039; 3040	4.87	.00189
HBA1; HBA2 (hemoglobin, alpha1, 2)	200169	4.87	.00776
GLT8D2 (glycosyltransferase 8 domain containing 2)	83468	4.848	5.21E-05
IL1R2 (interleukin 1 receptor, type II)	7850	4.832	.000357

Gene Name	Entrez Gene ID	Fold Change	P Value
CAMK2N1 (calcium/calmodulin-dependent protein kinase II inhibitor 1)	55450	4.8	7.50E-07
MFAP2 (microfibrillar-associated protein 2)	4237	4.693	.00116
LUM (lumican)	4060	4.669	.0087
DKK3 (dickkopf homolog 3 [<i>Xenopus laevis</i>])	27122	4.665	6.07E-05
ASPN (asporin [LRR class 1])	54829	4.606	.000668
PDGFD (platelet derived growth factor D)	80310	4.541	.000171
SLC16A6 (solute carrier family 16 (monocarboxylic acid transporters), member 6)	440459; 9120	4.535	.00119
CHI3L1 (chitinase 3-like 1)	1116	4.524	.00607
PDGFR1 (platelet-derived growth factor receptor-like)	5157	4.488	4.02E-06
RGC32 (response gene to complement 32)	28984	4.476	.000225
FLJ10781 (hypothetical protein FLJ10781)	55228	4.475	9.36E-05
PAG1 (phosphoprotein associated with glycosphingolipid microdomains 1)	55824	4.453	7.26E-07
HLA-DRA (major histocompatibility complex, class II, DR alpha)	3122	4.446	1.20E-05
CDC47L (cell division cycle associated 7-like)	55536	4.438	2.37E-07
PBK (PDZ binding kinase)	55872	4.412	.000345
TMEPAI (transmembrane, prostate androgen induced RNA)	56937	4.404	.00152
FNDC1 (fibronectin type III domain containing 1)	84624	4.309	.00272
FMOD (fibromodulin)	2331	4.305	1.94E-07
DSCR1 (Down syndrome critical region gene 1)	1827	4.297	4.11E-05
FJX1 (four jointed box 1)	24147	4.291	.00154
RSPO3 (R-spondin 3 homolog)	84870	4.28	.00531
ChGn (chondroitin beta1,4 N-acetylgalactosaminyltransferase)	55790	4.188	2.14E-05
CDC20 (CDC20-like protein)	991	4.18	.000132
BNC2 (basophilin 2)	54796	4.151	4.16E-05
MS4A7 (membrane-spanning 4-domains, subfamily A, member 7)	55244	4.125	.000607
FLJ10847 (hypothetical protein FLJ10847)	2533	4.111	.00455
FYB (FYN binding protein (FYB-120/130))	64167	4.096	.000345
LRAP (leukocyte-derived arginine aminopeptidase)	4016	4.086	.00697
LOXL1 (lysyl oxidase-like 1)	760	4.074	5.37E-05
CA2 (carbonic anhydrase II)	9509	4.068	3.81E-05
ADAMTS2 (ADAM metalloproteinase with thrombospondin type 1 motif, 2)	2115	4.064	.0019
ETV1 (ets variant gene 1)	27286	4.061	.000435
SRPX2 (sushi-repeat-containing protein, X-linked 2)	57381	4.049	.000489
RHOJ (ras homolog gene family, member J)	4316	4.037	1.17E-05

Abbreviations: Preg, tubal ectopic pregnancy; MSE, mid-secretory endometrium.

Table 4**Genes Downregulated in Preg Versus MSE**

Gene Name	Entrez Gene ID	Fold Change	P Value
CXCL13 (chemokine [C-X-C motif] ligand 13 [B-cell chemoattractant])	285708	-11.30	.000131
S100P (S100 calcium binding protein P)	54345	-10.88	.00185
TCN1 (transcobalamin I [vitamin B12 binding protein, R binder family])	57494	-8.40	.000396
SCGB2A2 (secretoglobin, family 2A, member 2)	148808	-7.81	.0107
CRISP3 (cysteine-rich secretory protein 3)	57494	-6.58	.000598
MALAT1 (metastasis associated lung adenocarcinoma transcript 1 [non-coding RNA])	145741	-5.78	7.97E-08
MT1M (metallothionein 1M)	283131	-5.68	.00464
C6orf141 (chromosome 6 open reading frame 141)	440552	-5.49	3.02E-06
EGFR (epidermal growth factor receptor [erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian])	1956	-5.41	4.98E-06
MGC42090 (hypothetical protein MGC42090)	148808	-5.03	3.87E-05
DKFZp761N1114 (hypothetical protein DKFZp761N1114)	135398	-4.76	9.42E-05
TncRNA (trophoblast-derived noncoding RNA)	4499	-4.76	.000113
LOC440552 (cytochrome c oxidase, subunit III)	378938	-4.63	3.73E-08
NLF1 (nuclear localized factor 1)	10321	-4.39	.00233
KIAA1238 (family with sequence similarity 80, member B)	4250	-4.29	.00158
DKFZp761N1114 (hypothetical protein DKFZp761N1114)	6947	-4.18	.000915
KIAA1238 (family with sequence similarity 80, member B)	6286	-4.07	.000662
SOX18 (SRY [sex-determining region Y]-box 18)	10563	-4.05	3.60E-06
LOC285708 (hypothetical protein LOC285708)	285708	-4.04	3.77E-08

Abbreviations: Preg, tubal ectopic pregnancy; MSE, mid-secretory endometrium.

Table 5

Real-Time Relative Quantification Transcript for Preg and MSE Samples

Gene of Interest ^a	Preg			MSE			P Value
	Median	Range		Median	Range	Fold Change Preg/MSE	
MMP11	0.22	0.0002–0.5		0.00007	0.00003–0.008	168.81	.0003
IGFBP1	3.11	0.012–204.85		0.66	0.033–1.414	69.96	.23
SST	0.78	0.000017–157.52		0.00013	0.00008–6.383	21.90	.23
PAL1	0.051	0.0067–0.831		0.0034	0.0016–0.022	20.98	.0025
INHBA	0.008	0.00007–0.086		0.000099	0.00005–0.003	19.41	.03
IL8	0.26	0.000003–6.59		0.00002	0.000004–0.75	16.31	.19
MMP7	1.51	0.02–30.29		0.26	0.007–0.72	15.05	.23
POSTN	0.12	0.00008–0.3566		0.004	6.74–0.05	14.11	.01
LCN2	9.55	0.23–43.46		1.61	0.43–2.03	10.26	.02
DKK3	0.24	0.004–0.96		0.003	0.00005–0.21	10.14	.001
CFH	5.36	1.48–14.68		0.0001	0.00007–4.23	6.45	.002
SFRP1	0.41	0.015–4.63		0.24	0.18–1.13	3.61	.54
Lumican	3.87	0.12–52.52		2.93	0.64–8.67	2.76	.67
TIMP3	1.85	0.77–46.98		2.81	0.99–9.57	2.49	.33
HLA-E	0.19	0.003–0.38		0.0007	0.000006–0.09	2.32	.001
S100A8	0.68	0.07–5.71		0.39	0.07–2.9	2.25	.4
FOXO1A	24.35	0.18–137.46		1.26	0.42–1.74	1.41	.02
ETS1	0.77	0.15–1.23		0.19	0.11–0.46	1.24	.005
IL-6	0.04	0.003–0.36		0.032	0.01–1.5	–2.50	.96
CFI	1.29	0.813–6.41		5.21	2.15–8.52	–2.70	.005
SP100	0.07	0.03–0.22		0.36	0.13–0.58	–3.67	.0006
DAF	1.15	0.16–3.48		15.99	4.62–36.36	–14.29	.001

Abbreviations: Preg, tubal ectopic pregnancy; MSE, mid-secretory endometrium.

^aSee text for gene name.

Table 6Comparison Between the Gene List From Sherwin et al¹² and Savaris et al (This Study)

Gene Name	Fold Change	
	Sherwin et al	Savaris et al (This Study)
Kynureninase (L-kynurenine hydrolase)	4.44	3.63
Serine (or cysteine) proteinase inhibitor, clade A (α -1 antiproteinase, antitrypsin), member 3a	46.14	3.2
BUB1 budding uninhibited by benzimidazoles 1 homolog β (yeast)	2.604	2.01
MMP7 (matrilysin, uterine)a	6.134	10.99
IL-1 β (interleukin 1, beta)	4.511	1.83
Complement component 4A	5.75	1.82
Growth arrest and DNA-damage-inducible, α	6.743	1.66
Cystathionase (cystathionine γ -lyase)	4.169	1.48
Small nuclear RNA activating complex, polypeptide 3, 50 kDa	4.202	1.18
IL-8 (interleukin 8)	3.304	5.314
Caspase 1, apoptosis-related cysteine protease (IL-1 β , convertase)	3.486	2.245
Cyclin B2	2.554	2.189
TPX2, microtubule-associated protein homolog (<i>Xenopus laevis</i>)	3.205	1.695
NDRG family member 2	0.287	1.660
Testican	3.296	1.588
Progestagen-associated endometrial protein (placental protein 14)	8.846	1.474
DnaJ (Hsp40) homolog, subfamily A, member 2	0.326	1.444
Sterol-C4-methyl oxidase-like	2.595	1.402
MMP23B	6.134	1.196
COX1/prostaglandin-endoperoxide synthase 1	0.361	1.343
FLJ44216 protein	0.358	1.219
Claudin 11 (oligodendrocyte transmembrane protein)	0.395	1.164
KIAA1975 protein similar to MRIP2	0.379	-1.064
Collagen, type IV, α 6	0.363	-1.179
SOD2, mitochondrial	5.389	-1.2
Hypothetical protein MGC20647	2.836	-1.222
IL-6 signal transducer (gp130, oncostatin M receptor)	3.445	-1.5
Gap junction protein, b 2, 26 kDa (connexin 26)	0.377	-1.56
Glycoprotein hormones, α polypeptide	3.457	-1.7
Hypothetical protein MGC3196	6.978	-1.164
Heparanase	6.234	n/a
MMP23A	6.133	n/a
Ribosomal protein L23	6.133	n/a
IL-1 receptor-like 1	6.014	n/a
Complement component 4B	5.75	n/a
Chemokine (C-X-C motif) receptor 4	5.47	n/a
Sortilin-related VPS10 domain containing receptor 3	4.861	n/a
Rai-like protein	4.078	n/a

Gene Name	Fold Change	
	Sherwin et al	Savaris et al (This Study)
Microsomal glutathione S-transferase 1	3.988	n/a
Placenta-specific 8	3.822	n/a
Complement component 3	3.677	n/a
Transforming growth factor β regulator 1	3.666	n/a
Peroxisome proliferative activated receptor, α	3.629	n/a
CCCTC-binding factor (zinc finger protein)-like	3.567	n/a
Discs, large homolog 7 (<i>Drosophila</i>)	3.542	n/a
Endothelin 3	3.307	n/a
Kinesin family member 9	3.208	n/a
Vascular endothelial growth factor C	2.987	n/a
Crystallin, α B	2.943	n/a
LIF (cholinergic differentiation factor)	2.836	n/a
Karyopherin α 2 (RAG cohort 1, importin α 1)	2.734	n/a
Hook homolog 2 (<i>Drosophila</i>)	2.699	n/a
ATPase, H ⁺ transporting, lysosomal 56/58 kDa, V1 subunit B, isoform 2	2.687	n/a
Cell division cycle 2, G1 to S and G2 to M	2.679	n/a
Sterol-C4-methyl oxidase-like	2.674	n/a
Diphtheria toxin receptor (heparin-binding epidermal growth factor-like growth factor)	2.609	n/a
SFRP4	0.242	n/a
DNA segment on chromosome 4 (unique) 234 expressed sequence	0.344	n/a
Adipose specific 2	0.379	n/a
Apolipoprotein A1	0.38	n/a
Hypothetical protein HT036	0.385	n/a

Abbreviation: n/a, not available in the significant gene list.