Global Gene Expression Profiling of Proliferative Phase Endometrium Reveals Distinct Functional Subdivisions

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Abstract

The human endometrium follows a predictable pattern of development during the proliferative phase. Endometrial thickness increases after day 3 and then plateaus at days 9 to 10 of the menstrual cycle despite continued high serum levels of estrogen. We hypothesized that proliferative phase endometrium undergoes more than simple estrogen responsive growth, rather it is characterized by complex time-dependent functional activities reflected in differential gene expression. Nine endometrial RNA samples from healthy participants were subjected to microarray analysis and 15 samples were used for quantitative real-time polymerase chain reaction. The samples were divided into early, mid, or late proliferative phase. The early proliferative phase showed higher expression of genes including transforming growth factor β 2, chemokine (C-C motif) ligand 18 (CCL18), and metallothionein 2A. The mid-proliferative phase was characterized by higher expression of heat shock proteins and implantation-associated genes including Indian hedgehog, secreted frizzled protein 4, and progesterone receptor. In the late proliferative phase, we identified increased angiotensin II receptor, type 2 and large decrease in expression of genes related to natural killer (NK) cell function. We demonstrate a unique gene expression signature at distinct time points within the proliferative phase. The early proliferative phase is characterized by tissue remodeling, angiogenesis, and modulation of inflammation; the mid-proliferative phase is characterized not only by proliferation in response to estrogens but also marks the onset of expression of genes required for endometrial receptivity and a dampening of estrogen responsiveness. In the late proliferative phase, changes in immune function and NK cells predominate. The proliferative phase is not simply a uniform period of estrogen responsive endometrial growth that can be considered as a single experimental time point when evaluating endometrial development; rather the proliferative phase is complex with differing functions and patterns of gene expression.

Keywords

early proliferative phase, mid-proliferative phase, late proliferative phase, endometrium

Introduction

The human endometrium is comprised of hormonally responsive glandular epithelium, stroma, and leukocytes.¹ The endometrium is responsive to sex steroid hormones, undergoes extraordinary growth in a cyclic manner, and is regenerated nearly 450 times in a woman's lifetime.² The primary function of the endometrium is to provide an attachment site and a source of nourishment to an early embryo. Therefore, the mechanisms that control and regulate the development of the endometrium are critical to the function of the uterus as a reproductive organ. To accomplish these diverse functions, it is reasonable to suspect that multiple mechanisms requiring the activation or repression of distinct genes must be operative at different times within the menstrual cycle.

The human endometrial cycle is divided into 2 dominant phases: the proliferative phase, which follows menstruation and precedes ovulation, and the secretory phase, which occurs postovulation.³ The proliferative phase is marked by the active

growth of stromal, epithelial, and vascular cells.⁴ While a significant amount of research has already been performed on the secretory phase of the menstrual cycle, specifically focusing on the window of implantation, much less is known about the proliferative phase.⁴⁻⁶ During the proliferative phase, endometrium grows from approximately 3 to 9 mm in height.⁷ These changes are regulated primarily by the ovarian steroid hormone, estradiol. During this phase, estrogens promote regeneration, proliferation, accompanied by changes in gene expression such as expression of the progesterone receptor,

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permitting the endometrium to respond to the progesterone produced in the secretory phase.^{8,9}

Recent studies have shown that endometrium increases in thickness and follows a predictable pattern of development during the proliferative phase.¹⁰ Endometrial height plateaus at days 9 to 10 of the menstrual cycle, despite high circulating estradiol levels.¹⁰ The proliferative phase is not simply a time of constant growth in response to estradiol, rather it is complex and the factors that regulate it are poorly understood. Here, we analyzed gene expression during distinct segments of the proliferative phase to gain a better understanding of endometrial growth and development.

Materials and Methods

Participants and Sample Collection

Endometrial samples were collected from 24 participants who underwent surgery in the proliferative phase between May 2010 and November 2010 for diagnosis or treatment of benign gynecological disorders. During surgery, no identifiable endometrial pathology or endometriosis was observed. Participants using hormonal medications were excluded. Endometrial biopsies were obtained using a Pipelle catheter (Cooper Surgical, Trumbull, Connecticut). The samples were divided according to the menstrual cycle as follows: early proliferative, days 1 to 3 ($n = 8$); mid proliferative, days 5 to 8 ($n = 8$); and late proliferative, days 11 to 13 ($n = 8$). Three samples from each phase were used for the array and 5 for quantitative polymerase chain reaction (qPCR). The mean age of the participants in the early proliferative group was 36.0 years, for the mid proliferative group was 34.7 years, and for the late proliferative group was 37.0 years. Approval was obtained from the Yale University School of Medicine Human Investigations Committee.

RNA Extraction

Total RNA from whole tissues was isolated using Trizol (Invitrogen, Carlsbad, California) per manufacturer's protocol. Total RNA was further purified with the RNeasy Kit purification (Qiagen, Valencia, California). The RNA was resuspended in 50 μ L of RNAase-free water, and its purity was assessed by both gel electrophoresis and spectrophotometry (A260/A280). All samples demonstrated ratios >1.8 . All purified products were stored at -80°C .

Microarray and Statistical Analysis

Prior to initiating the array, the quality of total RNA was analyzed on Agilent Bioanalyzer and RNA samples excluded if RNA integration number (RIN) was <7.0 . Purified RNA was analyzed using Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix, Santa Clara, California) which probes for 28 869 genes.

Data accrued in the microarray experiments were calculated using gene/transcript level expression. Partek Genomic Suite Software was used for microarray data analysis ([http://](http://www.partek.com/microarray)

www.partek.com/microarray). Robust Multichip Average (RMA) normalization method was performed.

A Student t test was used to identify those genes whose expression was statistically different between the 3 groups ($P < .05$). The cutoff range was a 2-fold change. Further validation was carried out using quantitative real-time (RT) PCR. Pathway analysis was performed using MetaCore software.

Quantitative RT-PCR

A total of 0.5 μ g of messenger RNA (mRNA) was reverse transcribed into complementary DNA (cDNA) using the iScript cDNA Synthesis kit at 46°C for 40 minutes in 20 μ L of reaction mixture (Bio-Rad Laboratories, Hercules, California). The resultant cDNA was stored at -20°C until further processing. Gene transcripts were amplified by RT-PCR using the Bio-Rad iCycler iQ system (Bio-Rad Laboratories). Primers were obtained from W M Keck Oligonucleotide Synthesis Facility, Yale University (as shown in Figure 1). Real-time PCR was performed using the iQ SYBR Green Supermix kit (Bio-Rad Laboratories). The reaction mixture included cDNA template (1 μ g), forward and reverse primers, RNase-free water, and the iQSYBRGreen Supermix, for a final reaction volume of 25 μ L. The thermal cycling conditions were initiated by uracil- N -glycosylase activation at 50°C for 2 minutes and initial denaturation at 95°C for 10 minutes, then 40 cycles at 95°C for 15 seconds, annealing at 60°C for 20 seconds. Melting analysis was performed by heating the reaction mixture from 74°C to 99°C at a rate of $0.2^{\circ}\text{C}/\text{s}$. Threshold cycle (C_t) and melting curves were acquired using the quantification and melting curve program of the Bio-Rad iCycler iQ system (Bio-Rad Laboratories). Only data with clear and single melting peaks were taken for further data analysis. Each reaction was performed in triplicates. The mRNA level of each sample was normalized to β -actin expression. Relative mRNA levels were presented using the formula $2^{-\Delta\Delta C_t}$.

Results

Differential Gene Expression Between Early, Mid-, and Late Proliferative Phase Endometrium

Proliferative samples were divided into 3 groups according to the menstrual cycle as follows: early proliferative (E) days 1 to 3 ($n = 5$), mid proliferative (M) days 5 to 8 ($n = 5$), and late proliferative (L) days 11 to 13 ($n = 5$).

To identify differential expression of candidate genes between E, M, and L, microarray analysis was initially performed. The top genes that had a 2-fold or greater fold change in expression and a P value $<.05$ were selected when comparing the groups.

Microarray Analysis

Early proliferative phase. Gene expression in this phase was characterized by a compilation of data comparing E versus M and L. The expression of 39 genes was significantly elevated

Figure 1. Quantitative RT-PCR comparing E, M, and L phases of the endometrial proliferative phase. Studies conducted with qRT-PCR display the fold change in gene expression for the 3 proliferative subphases. * $P < .05$ versus E was considered significant. E indicates early proliferative phase; M, mid-proliferative phase; L, late proliferative phase.

in the early proliferative phase by more than 2-fold. In E, we found higher expression of genes related to proliferation, cell differentiation, extracellular matrix modification, tissue remodeling, and angiogenesis.^{11,12} Specifically, transforming

growth factor $\beta 2$ (TGFB2), metallothionein 2A, perilipin 2, coagulation factor II (thrombin) receptor-like 2, and chemokine (C-C motif) ligand 18 (CCL18) were upregulated compared with the mid-proliferative phase (2.9-, 3.3- and 3.4-, 3.9-, and

Table 1. Microarray Analysis, Comparison of E Versus M

	Gene Name	Accession Number	P Value	Fold Change
TGFB2	Transforming growth factor, β 2	NM_001135599	.04	2.9
MT2A	Metallothionein 2A	NM_005953	.004	3.3
PLIN2	Perilipin 2	NM_001122	.04	3.4
F2RL2	Coagulation factor II (thrombin) receptor-like 2	NM_004101	.03	3.9
CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation)	NM_002988	.04	3.9

^a Table displays those genes with highest significant fold changes; n = 5 per group.

Table 2. Microarray Analysis, Comparison of M Versus E

	Gene Name	Accession Number	P Value	Fold Change
IHH	Indian hedgehog homolog (Drosophila)	NM_002181	.02	8.2
SFRP4	Secreted frizzled-related protein 4	NM_003014	.01	8.1
PGR	Progesterone receptor	NM_000926	.04	4.6
SNORD14E	Small nucleolar RNA, C/D box 14E	NR_003125	.03	4.2
GSTM1	Glutathione S-transferase mu 1	NM_000561	.02	4.0

^a Table displays those genes with highest significant fold changes; n = 5 per group.

Table 3. Microarray Analysis, Comparison of M Versus L^a

	Gene Name	Accession Number	P Value	Fold Change
HSPA1A	Heat shock 70 kDa protein 1A	NM_005345	.02	2.8
HSPA1B	Heat shock 70 kDa protein 1B	NM_005346	.02	2.9
SNORD14C	Small nucleolar RNA, C/D box 14C	NR_001453	.03	3.5
GSTM1	Glutathione S-transferase mu 1	NM_000561	.02	3.8
SNORD14E	Small nucleolar RNA, C/D box 14E	NR_003125	.01	6.0

^a Table displays those genes with highest significant fold changes; n = 5 per group.

3.9-fold, respectively; Table 1). These genes would be predicted to be critical for regeneration of the functionalis layer of the endometrium following the initial days after the menses.

Mid-proliferative phase. In the mid-proliferative phase, the expression of 62 genes was significantly elevated by more than 2-fold. In the M period, we observed the regulation of Indian hedgehog, the Wnt pathway including secreted frizzled protein 4 (SFRP4), and progesterone receptor that were upregulated in M versus E by 8.2-, 8.1-, 4.6-, and 4.2-fold, respectively (Table 2). These genes are generally involved in tissues requiring cell renewal including induction of cell proliferation, cell survival, and regulation of differentiation.¹³⁻¹⁶ These changes reflect the rapid growth and development of the functional layer of the human endometrium.

Although changes were not large, the retinoid X receptor β was significantly upregulated in M (fold change = 1.24 and $P = .03$). This is an important gene because it is involved in mediating the effects of retinoic acid known to be present at the site of decidualization and believed to be important for embryo implantation.¹⁷⁻²¹

Lastly, we observed the upregulation of heat shock 70 kDa protein 1A and B (HSPA1A/B) small nucleolar RNA, C/D box

14C and E (SNORD14C/E), and glutathione S-transferase mu 1 by 2.8-, 2.9-, 3.5-, 3.8-, and 6.0-fold (Table 3).

Late proliferative phase. We identified 32 genes expressed at a significantly greater level in the late proliferative phase. The late proliferative phase is characterized as the period that precedes ovulation. At this point of the menstrual cycle, there is a growth plateau and many of the events that will be essential for embryo implantation are thought to occur. Hence, we observed upregulation of genes such as angiotensin II receptor type 2 (AGTR2) that inhibit cell growth, remodel extracellular matrix, and are involved in cellular differentiation.²² Other genes that were upregulated in this period included lipoma HMGIC fusion partner, chromosome 9 open reading frame 131 (C9orf131), small nucleolar RNA, H/ACA box 23 (SNORA23), and cysteine-rich transmembrane Bone Morphogenic Protein (BMP) regulator 1 (chordin-like; CRIM1). These genes were upregulated by 3.4-, 3.0-, 2.2-, 2.2-, and 2.9-fold (Table 4). The induction of these nuclear targeting genes is likely central to the cyclical changes observed in the human menstrual cycle.

We also observed the upregulation of SFRP4, AGTR2, solute carrier family 27 (fatty acid transporter, adenylate cyclase-activating polypeptide 1 (pituitary), and ADAM

Table 4. Microarray Analysis, Comparison of L Versus M^a

	Gene Name	Accession Number	P Value	Fold Change
AGTR2	Angiotensin II receptor, type 2	NM_000686	.04	3.4
LHFP	Lipoma HMGIC fusion partner	NM_005780	.03	3.0
C9orf131	Chromosome 9 open reading frame 131	NM_203299	.01	2.2
SNORA23	Small nucleolar RNA, H/ACA box 23	NR_002962	.009	2.2
CRIMI	Cysteine-rich transmembrane BMP regulator 1 (chordin-like)	NM_016441	.02	2.1

^a Table displays those genes with highest significant fold changes; n = 5 per group.

Table 5. Microarray Analysis, Comparison of E Versus L^a

	Gene Name	Accession Number	P Value	Fold Change
KIR2DL3	Killer cell immunoglobulin-like receptor, 2 domains, I	NM_015868	.04	7.0
KLRC3	Killer cell lectin-like receptor subfamily C, member 3	NM_002261	.04	5.9
SH2D1B	SH2 domain containing 1B	NM_053282	.04	5.2
LEFTY2	Left-right determination factor 2	NM_003240	.04	4.1
F2RL2	Coagulation factor II (thrombin) receptor-like 2	NM_004101	.04	3.4

^a Table displays those genes with highest significant fold changes; n = 5 per group.

Table 6. Microarray Analysis, Comparison of L Versus E^a

	Gene Name	Accession Number	P Value	Fold Change
SFRP4	Secreted frizzled-related protein 4	NM_003014	.02	5.2
SLC27A6	Solute carrier family 27 (fatty acid transporter), me	NM_001017372	.02	4.8
ADCYAP1R1	Adenylate cyclase activating polypeptide 1 (pituitary)	NM_001118	.04	4.6
AGTR2	Angiotensin II receptor, type 2	NM_000686	.03	3.6
ADAMTS19	ADAM metalloproteinase with thrombospondin type 1 motif,	NM_133638	.04	3.0

^a Table displays those genes with highest significant fold changes; n = 5 per group.

metalloproteinase with thrombospondin type 1 motif. Interestingly, several genes associated with uterine natural killer (NK) cell function (KIR2DL3 and KLRC3) showed decreased expression in the late proliferative phase (Tables 5 and 6).

Quantitative RT-PCR

In order to validate some of our microarray results, quantitative RT-PCR (qRT-PCR) was performed on all 15 samples collected. Results were analyzed using analysis of variance. Genes reported showed significant differences in steady state mRNA levels with a *P* value <.05. The genes discussed above which were significant in the microarray were confirmed using qRT-PCR.

Several genes were dramatically and significantly increased in the early proliferative phase. Additional genes that had higher expression during the early proliferative phase of the menstrual cycle are shown in the Figure 1. Specifically, Vanin 1 (VNN1), Progesterone associated endometrial protein (PAEP), Fibrinogen beta chain (FGB) and Inhibin beta A (INHBA), matrix metalloproteinase 1 (MMP-1)-1, interleukin 8 (IL-8), FGB, and INHBA were expressed 40-, 33-, 32-, 30-, 725-, 279-, 217-, and 200-fold times higher in E than the latter parts of the proliferative phase.

Pathway analysis of the microarray results revealed a mid-proliferative phase increase in hedgehog and progesterone receptor-mediated signaling. In the late proliferative phase, we saw a decrease in immune response genes related to NK cell function.

Discussion

Endometrial growth and function is complex and differs during distinct windows of the proliferative phase of the menstrual cycle. Healing is followed by rapid growth, however, showing a plateau around cycle day 9 or 10.¹⁰ We hypothesized that the diverse range of growth during the proliferative phase occurs because of gene activation and/or suppression during this stage of the menstrual cycle. While attention has been given to the different subdivisions of the secretory phase, few studies have interrogated gene expression during subdivisions of the proliferative phase.

The early proliferative phase follows menstruation and has been described as a wound healing-like process characterized by an inflammatory response.²³ These phenomena occur even in the absence of steroid hormones, indicating that other regulatory mechanisms are involved in this process.²³ In the early proliferative phase, we saw a higher expression of genes related to proliferation, cell differentiation, extracellular matrix modification, tissue remodeling, and angiogenesis.¹¹ Similarly, we

Table 7. Primers Used for Validation by qRT-PCR

Progesterone receptor	FW: TAGGGCTTGGCTTTCATTTG RV: TGGAAGAAATGACTGCATCG
Indian Hedgehog	FW: GCGCGGTGGACATCACCACA RV: CGGCCGAGTGCTCGGACTTG
Secreted frizzled protein 1 FW	FW: GGGACGTCTGCATCGCCATG RV: CACAGGGAGGACACACCGTTGT
Secreted frizzled protein 4 FW	FW: GACGAGCTGCCTGTCTATGACC RV: GTACCATCATGTCTGGTGTGATGTC
Adenylate cyclase-activating protein	FW: CTCCGAGCCACCGAAGTCT RV: GCCCTGCTGGTCTATGGGATA
Inhibin β 1	FW: GTTTGCCGAGTCAGGAACAGCCA RV: GCACGCTCCACCACTGACAGG
Indoleamine 2,3 dioxygenase	FW: GGGGCAGTGCAGGCCAAAGC RV: GCATGTCCTCCACCAGCAGTCTG
Fibrinogen β chain	FW: TGGAGGTGGCTATCGGGCTCG RV: AGGACACAACACCCCCAGGTCTG
Deleted in malignant brain tumor	FW: CGACGCCCAGTCCAGACACG RV: CCCAGCTGCCTGCAGACCAC
Connective tissue growth factor	FW: GTCCGCGTCGCCTTCGTGGTC RV: AGGGAGCACCATCTTTGGCGGTG
Replication factor C activator 1	FW: GGCCCATTTGATGTTGCCCGG RV: TGTCACCCCCTGCTGCTACAGG
Angiotensin II receptor type 2	FW: CGTCCCAGCGTCTGAGAGAACG RV: CACACTCCTTCAAAATTCAGGCTGC
Progestagen endometrial protein	FW: CCCTGCCCAGGCACCTATGGTA RV: GAGGTGAGCCAGGAGGCAGGACC
Matrix metalloproteinase 8	FW: TGGCCATTCTTTGGGGCTCGC RV: GGATGCCTTCTCCAGAAGTACCTGT
Matrix metalloproteinase 1	FW: GGGGAACCCTCGCTGGGAGC RV: GTTGTCCCGATGATCTCCCCTGACA
Heat shock 70 kDa 1B	FW: GTCATCTCGTGGCTGGACGCC RV: CTTGAGTCCCAACAGTCCACCTCA
Interleukin 8	FW: TGTGAAGGTGCAGTTTTGCCAAGG RV: GTTGGCGCAGTGTGGTCCACTC
Vanin 1	FW: GCCCAATGCCACCCTAACACCAG RV: TGGGGTCTGGCCAAATCTGTTACG
Secreted phosphoprotein 1	FW: AATCTCCTAGCCCCACAGAATGCTG RV: TCGGTTGCTGGCAGGTCCGT
β -actin	FW: CGTACCACTGGCATCGTGAT RV: GTGTTGGCGTACAGGTCTTTG

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.

observed higher expression of genes related to immunomodulation, cell recruitment, inflammation, and generation of an immune response.²⁴⁻²⁸ Both TGFB2 and (C-C motif) ligand 18 (CCL18) are the examples of those genes that were upregulated in the early phase and would be expected to be involved cell proliferation and immune responses.

The mid-proliferative phase is characterized by the peak of proliferation and angiogenesis. Alterations in gene expression in this period could compromise these processes which in turn may be critical for subsequent embryo implantation during the secretory phase.²⁹ Distinct genes that modulate growth and induce cell proliferation were upregulated in the mid-proliferative phase. Surprisingly, genes normally associated with implantation are expressed here as well. The foundation of the implantation window is initiated in the mid-proliferative phase. Among those genes, we observed changes in Indian hedgehog,

SFRP4 a modulator of Wnt-signaling pathway,³⁰⁻³² and the progesterone receptor. Alterations as early as the mid-proliferative phase may affect the implantation window.

Genes that were higher at the mid-proliferative phase also included genes related to function of the steroid receptor proteins.^{33,34} Increased levels of heat shock proteins 1A and 1B, which act as chaperone proteins modulating sex steroid receptors, may explain the halt of endometrial growth in the presence of high circulating estrogen levels.

The late proliferative phase precedes ovulation. At this point of the menstrual cycle, embryo implantation necessitates a role of the immune response.³⁵ Natural killer cells play an important task in the early phase of the innate immune response and are essential for embryo attachment.³⁶ However, they have been shown to be present in nonpregnant uterus, and their number changes during the menstrual cycle, suggesting that

endometrial stromal cells may exert immune regulation of uterine NK cell proliferation, apoptosis, and differentiation.³⁷ We identified lower expression of genes such as killer cell immunoglobulin-like receptor 2 domains 1 and killer cell lectin-like receptor subfamily C, member 3. These genes are related to NK cell function and indicate a dampening of NK cell immune response at this stage of the menstrual cycle. Immune modulation in preparation for embryo implantation appears to be a significant function of the late proliferative phase.

The proliferative phase has discrete subdivisions with markedly different patterns of gene expression reflecting distinct functions. We posit that the changes in gene regulation are critical for endometrial growth, angiogenesis, and immune responses. The proliferative phase is complex and cannot be simply considered a uniform period of growth in response to estrogen. The foundation of the implantation window begins in this phase, and it is likely that implantation defects may also have their origin here as well. Future studies on endometrial differentiation should take into account the changing nature of the proliferative phase endometrium.

Declaration of Conflicting Interests

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

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