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Research Article

Oestrogen-modulated gene expression in the human endometrium

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Abstract. To identify key regulatory mechanisms in the growth and development of the human endometrium, microarray analysis was performed on uncultured human endometrium collected during menstruation (M) and the late-proliferative (LATE-P)-phase of the menstrual cycle, as well as after 24 h incubation in the presence of oestradiol (17β-E2). We demonstrate the expression of novel gene transcripts in the human endometrium. i.e. mucin-9, novel oestrogen-responsive gene transcripts, i.e. gelsolin and flotillin-1, and genes known to be expressed in human endometrium but not yet shown to be oestrogen responsive, i.e. connexin-37 and TFF1/pS2. Genes reported to be expressed during the implantation window and implicated in progesterone action, i.e. secretoglobin family 2A, member 2 (mammaglobin) and homeobox-containing proteins, were up-regulated in uncultured LATE-P-phase endometrium compared to M-phase endometrium. Some gene transcripts are regulated directly by 17β-E2 alone, others are influenced by the in vivo environment as well. These observations emphasise that the regulation of endometrium maturation by oestrogen entails more then just stimulation of cell proliferation.

Key words. Steroid hormones; cell differentiation; extracellular matrix remodelling; angiogenesis; physiology.

During the menstrual cycle, the growth and differentiation of the human endometrium is controlled by oestrogen and progesterone. After shedding of the functional layer of the endometrium during menstruation, a series of events occurs in the proliferative phase of the menstrual cycle, aimed at regeneration of damaged endometrium, subsequently followed by the creation of a new functional layer. A variety of biological processes occurs during human endometrium maturation in the proliferative phase, mainly governed by oestrogen. i.e. cell proliferation [1], cell differentiation, extracellular matrix remodelling, angiogenesis and vasculogenesis [2]. However, the genes, molecular mechanisms and pathways by which oestrogen regulates these events are only partly understood, mainly due to the complexity and the large number of factors acting in concert. Although endometrium physiology has been an important subject of research, most attention has focussed on determining the expression of gene transcripts and proteins during the implantation window [3–6]. Recent studies demonstrated that endometrium receptivity declines when primed inappropriately, i.e. exposed to either high [7] or low doses of oestrogen [8, 9] prior to ovulation.

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The aim of this study was to unravel key mechanisms that underlie oestrogen-controlled maturation in the human endometrium. Two approaches were taken to increase our current understanding of the role of oestrogen in regulating gene expression in the human endometrium. In the first approach, we compared the gene expression profiles of endometrium tissue in the menstrual phase (M phase) that has not been exposed to increased peripheral concentrations of follicular oestrogens with endometrium tissue that has been exposed to increased concentrations of follicular oestrogens for several days (late proliferative, LATE-P phase). In the second approach, the gene transcripts directly modulated by oestrogens were discriminated from those that require in vivo oestrogen priming. To this end, we compared the expression profiles of endometrium explants cultured in the absence or presence of oestradiol (17β-E2) for 24 h.

Material and methods

Human endometrial tissue

All patients were 20–45 years old, had regular ovulatory cycles and were not receiving hormonal treatment. Endometrium was collected by pipelle biopsy or from hysterectomy specimens. Endometrial biopsies were obtained with a Pipelle catheter (Unimar, Prodimed, Neuilly-Enthelle, France) under sterile conditions, from female volunteers who came to the clinic for a diagnostic laparoscopy as part of their fertility work-up, or for a sterilisation procedure. Indications for hysterectomy were excessive menstrual bleeding or myomas. The endometrium was inspected macroscopically by the pathologist, and normal-appearing endometrium was scraped gently from the surface of the uterine cavity with a sterile surgical blade. Part of the endometrial tissue used for the study was fixed in 4% formaldehyde for histological examination. Endometrium biopsies were dated according to the microscopic criteria of Noyes et al. [10], and the dating was adjusted finely according to clinical information with respect to the start of the last menstrual period whenever available. All women gave their written informed consent, according to a protocol approved by the Medical Ethical Committee of the Academic Hospital Maastricht.

A total of 24 biopsy samples were obtained during the M phase, i.e. cycle days (CD) 2–5 and LATE-P phase, i.e. CD 11–14 (prior to ovulation). Of the 24 biopsies, 4 were used for microarray studies and 20 were used for validation with real-time PCR analysis. Tissue was transported to the laboratory in DMEM/Ham's F12 medium on ice. A portion of each sample was fixed in 10% buffered formalin for histology evaluation. The samples that were used to determine the in vivo oestrogen-regulated genes (designated as uncultured) were immediately placed in lysis buffer and stored at –70 °C. A part of the explants were cultured on Millicell culture inserts as described previously (Punyadeera et al., J Steroid Biochem Mol Biol, 2004, in press).

Human endometrial explant cultures and RNA extractions

Human endometrium tissue was cut into pieces of 2–3 mm^3. Twenty-four explants were applied on Millicell-CM culture inserts (pore size 0.4 μm, 30 mm diameter; Millipore, ■■, France) in six-well plates containing phenol red-free DMEM/Ham's F12 medium (1.2 ml) (Life Technologies, Grand Island, N. Y.). The medium was supplemented with L-glutamine (1%), penicillin and streptomycin (1%, P/S) and this was used in all stages of explant preparation. Cultures were performed for 20–24 h. Previous experiments have shown that collagenase activity remains low in proliferative endometria during the first 24 h of culture [12], and that the tissue viability is not affected after 24 h culture [13]. The treatments included: (i) control (0.1% ethanol) and (ii) 17β-E2 (1 nM). 17β-E2 was a gift from Organon N.V. (Oss, The Netherlands).

Total cellular RNA was extracted from explants using the SV total RNA isolation kit (Promega, Madison, Wis.) according to the manufacturer's protocol, with slight modifications. The concentration of DNase-1 during DNase treatment of the RNA samples was doubled and the incubation time was extended by 15 min to completely remove genomic DNA. Total RNA was eluted from the column in 50 μl RNase-free water and stored at –70 °C until further analysis. The quality of the RNA samples was determined with the Agilent bioanalyzer 2100 lab-on-a-chip (Agilent, ■■, ■■). All the samples analysed gave 28S to 18S ratios higher than 1.5. A PCR for a housekeeping gene, GAPDH, was performed to confirm that the RNA samples were free of genomic DNA.

Affymetrix gene chip micro-arrays

The RNA samples were pooled according to the phase of the menstrual cycle and to the treatment conditions, i.e. two RNA samples from the M phase and two RNA samples from the LATE-P phase were pooled. From pooled RNA, cRNA was generated and labelled with biotin according to the Affymetrix protocol (Santa Clara, ■■, ■■). cDNA was hybridised to the Affymetrix HU-133A chips, which contain approximately 22,000 human oligonucleotide probe sets, including 68 controls. The chip hybridisations were carried out in triplicate. After washing, the chips were scanned and analysed using the MicroArray suite MAS5. A detailed description of the Affymetrix chip content is available at the NetAffy analysis web page (http://www.affymetrix.com/analysis/index.affx).

Micro array data analysis

Following Gene chip data quality control, data files (.EXP, .DAT, .CEL) generated by MAS5 were transferred by FTP to the server housing the Rosetta Resolver Gene Expression Data Analysis System. Resolver uses its Affymetrix gene chip error model to transform the raw data into a processed form that can be used in various expression analyses. Rosetta Resolver allows normalisation of sample data of triplicate hybridisations using one-way analysis of variance (ANOVA) [14]. After data transformation, the scatter plots of log relative intensities of all detected gene transcripts had slopes of around 1, indicating that hybridisation efficiency was similar for the arrays in both groups and array comparisons could be made.

The use of microarrays results in a massive amount of data, which require special tools to filter and extract the relevant information. By combining the fold changes or log ratios and the p value, we generated a so-called significance code, which simplified the selection and extraction of genes of interest, especially when analysing various conditions. The significance code assigned to the genes was based on ANOVA-retrieved p values, and up- or down-regulation compared to the untreated samples. A significance code of +1 was used for genes with $p < 0.01$ and log ratio >0 (stimulated or up-regulated); a significance code of –1 was used for genes with $p < 0.01$ and log ratio <0 (inhibited or down-regulated). Genes which did not show a significant regulation received the significance code of 0. Data were then exported from Rosetta Resolver to Spot fire decision site 7.1 (Spotfire, Göteborg, Sweden), in which gene sets of interest were visualised and subsequently selected. Figure 1 presents an illustrative example of the significance code approach when comparing more than two treatments. Using this strategy, gene transcripts that are modulated under three different treatment conditions could easily be identified.

The genes were annotated using the Affymetrix annotation data base [15]. After annotation, the genes were allocated to 1 of 18 functional categories based either on the biological process or their cellular or molecular functions.

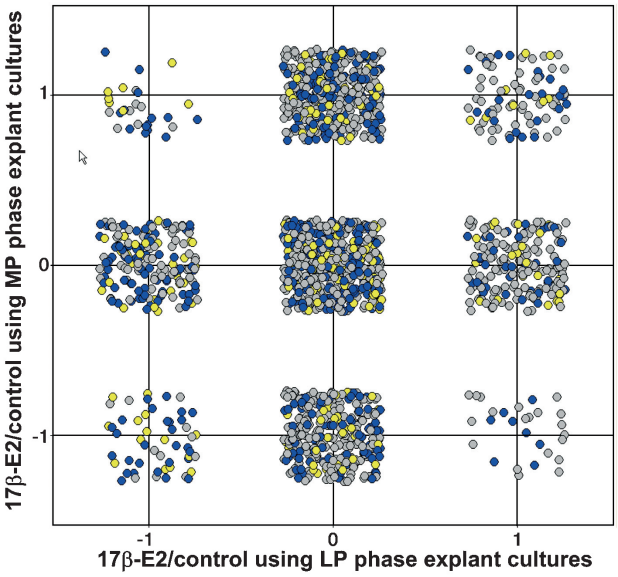

Figure 1. An illustrative example of the significance code when comparing more than two treatment conditions. The coordinates –1, 0 and +1 present the assigned significance codes (detailed description in Materials and methods). The y-axis represents the ratio of 17β-E2/control for M-phase endometrium explant cultures (treatment 1); the x-axis represents the ratio of 17β-E2/control for LATE-P-phase endometrium explant cultures (treatment 2); the z-axis (colours) represents the ratio of uncultured LATE-P-phase/uncultured M phase (treatment 3). The yellow dots represent the up-regulated genes; blue dots represent down-regulated genes and the grey dots represent genes that are not regulated. If the coordinates are –1 and –1 and the dot is blue, this would represent the down-regulated genes by 17β-E2 treatment in both M- and LATE-P-phase endometrium tissue and expressed lower in LATE-P-phase uncultured endometrium compared to M-phase uncultured endometrium.

Real-time PCR analysis

Newly identified gene transcripts in the endometrium that showed more than a twofold stimulation (or down-regulation), and gene transcripts with documented biological relevance to human endometrium physiology were selected for validation using real-time PCR analysis. Samples from additional experiments were analysed using endometrium tissue obtained throughout the proliferative phase to validate the microarray results. Total RNA (1 µg) was incubated with random hexamers (1 µg/µl, Promega) at 70 °C for 10 min. The samples were chilled on ice for 5 min. To this mixture, a reverse transcriptase (RT)-mix consisting of 5× RT buffer (4 µl), 10 mM dNTP mix (1 µl) (Pharmacia, Uppsala, Sweden), 0.1 M DTT (2 µl) (Invitrogen, ▮▮, Calif.) and superscript II reverse transcriptase (200 U/µl) (Invitrogen, California, USA) was added and the samples were incubated at 42 °C for 1 h, after which the reverse transcriptase was inactivated by heating the samples at 95 °C for 5 min. The cDNA was stored at –20 °C until further use. In each real-time PCR reaction 50 ng of cDNA template was used.

Primers and probes were purchased from Perkin-Elmer Applied Biosystems as pre-developed assays. Human cyclophylin A was selected as an endogenous RNA control to correct for the differences in the amount of total RNA added to each reaction. Uncultured human endometrium tissues were included as positive controls. All PCR reactions were performed using an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, Calif.). The thermal cycling conditions comprised an initial decontamination step at 50 °C for 2 min, a denaturation step at 95 °C for 10 min and 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. Experiments were performed for each sample in duplicate. Quantitative values were obtained from the threshold cycle num-

ber (Ct) at which the increase in the signal associated with exponential growth of PCR products is first detected with the ABI Prism 7700 sequence detector software (Perkin-Elmer, Foster City, Calif.). The relative expression of the target gene in the human endometrium tissue was calculated by $1/2^{\Delta Ct}$ ($\Delta C_t = C_{t\,target} - C_{t\,cyclophilin\,A}$). The fold-change in expression was calculated using the ΔΔ Ct method, with cyclophylin A mRNA as an internal control [16]. For detailed description of the procedure please refer to the ABI user manual: http://www.uk1.unifreiburg.de/core/facility/tagman/userbulletin_2.pdf).

Statistical analysis of quantitative real-time PCR data

Statistical tests were carried out using the SPSS 10 (SPSS, Chicago, Ill.) statistical analysis package. The non-parametric unpaired Mann-Whitney U test at a confidence level of 95% was employed to analyse the real-time PCR data generated from uncultured LATE-P-phase endometrium tissue and uncultured M-phase endometrium tissue. The effects of 17β-E2 on cultured explants were analysed using the non-parametric paired Wilcoxon signed-rank test at a confidence level of 95%.

Results

Gene expression was studied in the human endometrium tissue, collected either directly from the M phase and LATE-P phase (uncultured) or after 24 h culture in the presence of 17β-E2 or vehicle. The differences in gene expression between groups (i.e. LATE-P phase versus M phase, and 17β-E2 versus control for both M) and LATE-P-phase explant cultures) were calculated, and gene transcripts with a significant decrease or increase of twofold or more were used.

Validation of array data

The expression of six genes was validated in the original RNA samples used for the array hybridisations (table 1). In addition, eight additional samples were collected and analysed to evaluate the biological variation between women for the expression of the six genes validated from the array hybridisations (table 1). The genes were selected based on known 17β-E2 responsiveness, i.e. progesterone receptor (PR), trefoil factor-1 (TFF1/pS2), and cyclin A, on novelty, i.e. mucin-9, and on documented involvement in endometrial function, i.e. connexin-37, matrix metalloproteinase-1 (MMP-1), cyclooxygenase-2 (COX-2) and 17β-HSD type 2. In addition, two gene transcripts were selected that have not been previously documented to be expressed in the endometrium, i.e. secretoglobin 1D, member 2 or lipophilin B, and regarded as being regulated by progesterone only, i.e. secretoglobin 2A, family member 2, mammaglobin A.

The findings of the array results could be confirmed with real-time PCR (table 1). However, when screening additional biopsies, only the expression of secretoglobulin 2A, family member 2 and MMP-1 was significantly higher in LATE-P-phase compared with M-phase endometrium. The differences detected with the arrays for PR, cyclin A1, connexin-37 and mucin-9 could not be confirmed in the extra set of tissues. This was due to the fact that in the array hybridisations, one sample was expressed higher in LATE-P-phase endometrium, and the other not.

In the cultured endometrial explants, however, the response to 17β-E2 as detected in the array hybridisations could be confirmed for all selected genes (table 2). In the independent set of experiments, six of the seven tested genes were responsive to 17β-E2 (fig. 2). Only the secretoglobin family 2A, member 2 was not responsive to 17β-E2 in the additional set of experiments.

Table 1. Validation of microarray data for the uncultured M- and LATE-P-phase endometrium tissue. Real-time PCR was performed on the same samples as used for the array hybridisations (columns 1 and 2), as well as on an additional set of M- and LATE-P-phase endometrium samples (columns 3–5). Validation of array data are presented as ratios of LATE-P-phase/M, and data on the additional set are presented as relative expression levels (± SE).

Gene name	Array data (LP/M) phase (uncultured)	Real-time PCR (LP/M) phase (uncultured)	Additional experiments		
			M phase (uncultured) (mean ± SE)	LATE-P phase (uncultured) (mean ± SE)	
Progesterone receptor	9.1	3.4	0.23 ± 0.08	0.38 ± 0.15	p = NS
Cyclin A1	3.4	2.2	0.015 ± 0.003	0.021 ± 0.007	p = NS
Secretoglobin family 1D, member 2	4.9	2.8	0.111 ± 0.008	0.725 ± 0.402	p < 0.01
Mucin-9	36.3	10.3	0.30 ± 0.20	0.09 ± 0.04	p = NS
Matrix metalloproteinase-	10.01	0.002	0.66 ± 0.49	0.0004 ± 0.0002	p < 0.01
Connexin-37	10.0	3636	0.019 ± 0.0089	0.015 ± 0.0053	p = NS

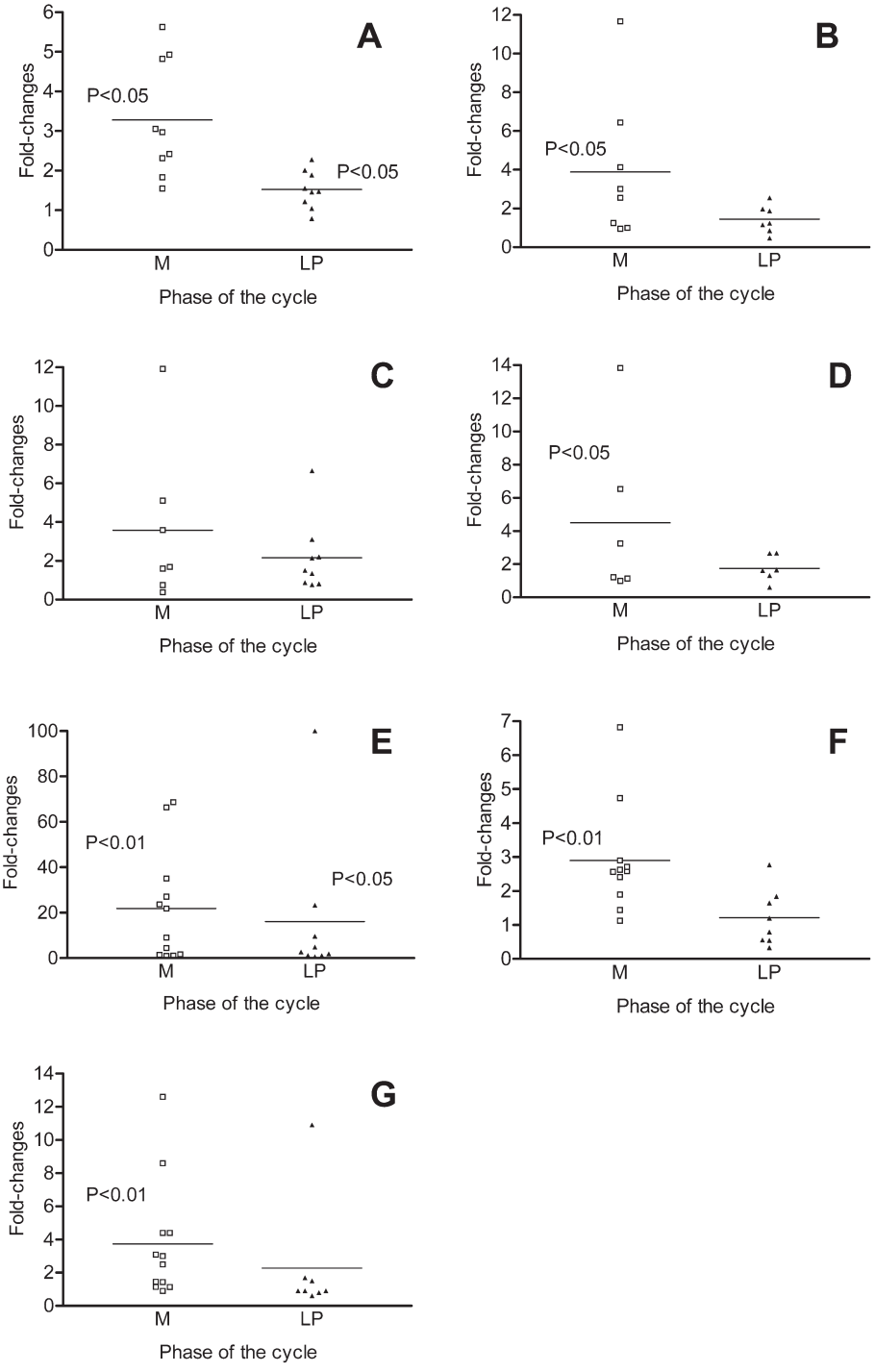

Figure 2. Validation of the microarray findings with quantitative real-time PCR analysis in an independent set of experiments. Endometrium tissue collected from M phase (□, n = 9 or 10) and LATE-P phase (▲, n = 8) was cultured in the presence of 17β-E2 or vehicle. Values are expressed as fold-changes relative to the vehicle and the average values are presented by a horizontal bar. (*A*): Progesterone receptor. (*B*): Cyclin A1. (*C*): Secretoglobin family 2A, member 2. (*D*): Secretoglobin family 1D, member 2. (*E*): Trefoil factor-1. (*F*): Cyclooxygenase-2. (*G*): Connexin-37.

Table 2. Validation of microarray data for the cultured M- and LATE-P-phase endometrium explants in the presence or absence of 17β-E2.

Gene name	Array data M phase (cultured)	Real-time PCR M phase (cultured)	Array data LP phase (cultured)	Real-time PCR LP phase (cultured)
Progesterone receptor	3.0	2.0	1.1	1.3
Cyclin A1	3.5	5.9	1.1	1.6
Secretoglobin family 1D, member 2	8.9	6.4	1.9	2.1
Secretoglobin family 2A, member 2	3.7	6.7	1.6	1.4
Trefoil factor 1	7.8	50.0	1.2	5.0
Cyclooxygenase-2	3.5	6.5	1.2	1.3
17•-HSD Type 2	2.5	2.6	1.4	1.3
Connexin-37	3.5	3.5	0.9	2.2

Values are presented as fold changes in the induction by 17β-E2 (±SE) compared to the control.

Long-term oestrogen exposure (uncultured)

Of the 22,000 transcripts present on the HU133A chip, 282 transcripts were found to be up-regulated in LATE-P phase versus M phase and 512 transcripts were down-regulated in the LATE-P-phase versus the M-phase endometrium biopsies. In tables 3 (parts A and B), the ten most regulated genes are presented.

Short-term oestrogen exposure (cultured)

To distinguish the genes that are directly regulated by 17β-E2 from those affected by the in vivo uterine environment, the differential gene expression profiles were determined for endometrium tissue fragments that were incubated with or without 17β-E2 for 24 h. After incubation of M-phase endometrium explants with 17β-E2, 148 transcripts were found to be stimulated and 45 transcripts were inhibited (for the top ten regulated genes see table 3, parts C and D. In contrast, in LATE-P-phase endometrium cultures, the expression of a lower number of genes was changed: only 12 transcripts were stimulated and 4 transcripts were inhibited. Table 3, parts E and F present the most regulated genes.

The expression of six genes was stimulated by 17β-E2 in both M- and LATE-P-phase endometrium explants indicating gene transcripts that are directly regulated by oestrogens, whereas the expression of two genes was inhibited by 17β-E2 in both M- and LATE-P-phase endometrium explants (table 4).

Thirteen genes were stimulated by 17β-E2 in cultures of M-phase endometrium ànd up-regulated in vivo (LATE-P phase versus M). Seven genes were inhibited by 17β-E2 in vitro and down-regulated in vivo (LATE-P phase vs. M) (table 5).

In contrast, 15 genes were stimulated by 17β-E2 in cultures of M-phase endometrium and down-regulated in vivo (LATE-P phase vs. M). Three gene transcripts were inhibited by 17β-E2 in vitro and up-regulated in vivo (LATE-P phase vs. M) (table 5, bold font).

Discussion

To enhance our current understanding of the role of oestrogen in the development of the human endometrium during the proliferative phase of the menstrual cycle, gene expression profiles were compared between uncultured menstrual (M) phase and uncultured late proliferative phase (LATE-P-phase) endometria, and between M-phase and LATE-P-phase endometrium tissue explants after short-term incubation with 17β-E2.

This study revealed the expression of a number of novel gene transcripts in the human endometrium (i.e. mucin-9, dipeptidyl peptidase VI). In addition, we found novel genes that were oestrogen responsive (gelsolin, flotillin-1, TUWD12), and genes known to be expressed in human endometrium (TFF1/pS2, connexin-37, placental alkaline phosphatase) but not yet shown to be oestrogen responsive. Based on the observed in vitro responses and in vivo differences in M and LATE-P phase, some gene transcripts are apparently either regulated directly by 17β-E2, or by 17β-E2 in concert with the changing uterine environment. Genes reported to be expressed during the implantation window and implicated in progesterone action, i.e. secretoglobin family 2A, member 2 (mammaglobin) and homeobox containing proteins, were up-regulated in uncultured LATE-P-phase endometrium compared with uncultured M-phase endometrium, implying that these genes may also be regulated by oestrogens. These observations emphasise that the regulation of endometrium maturation by oestrogen entails more then just stimulating cell proliferation and growth.

To study the effects of oestrogens on human endometrium, an explant culture system was used. The advantage of using explant cultures over other systems is the fact that no enzymatic manipulations are used, leaving the original structure intact. To find oestrogen-responsive genes, we had to select tissues that had not been exposed to oestrogens produced by the ovaries. Only menstrual endometrium meets this criterion. The findings

Table 3. The ten most up-regulated gene transcripts in uncultured LATE-P-phase endometrium compared with uncultured M-phase endometrium (A), down-regulated gene transcripts in uncultured LATE-P-phase endometrium compared with uncultured M-phase endometrium (B), up-regulated (C) and down-regulated (D) gene transcripts in M phase endometrium in the presence of 17β-E2, and up-regulated (E) and down-regulated (F) gene transcripts in LATE-P-phase endometrium in the presence of 17β-E2.

A Gene name	Symbol	Up	GenBank No.	**B** Gene name	Symbol	Down	GenBank No.
Oviductal glycoprotein 1 (mucin 9, oviductin)	OVGP1	36.3	NM_002557.1	Inhibin, beta A	INHBA	–100	M13436.1
Olfactomedin 1	OLFM1	20.4	NM_006334.1	Interleukin 8	IL8	–100	NM_000584.1
Kinesin family member 4A	KIF4A	13.2	NM_012310.2	Matrix metalloproteinase 1 (interstitial collagenase)	MMP1	–100	NM_002421.2
Troponin C, slow	TNNC1	13.2	AF020769.1	Matrix metalloproteinase 3 (stromelysin 1)	MMP3	–93.3	NM_002422.2
Kinesin family member 20A	KIF20A	12.9	NM_005733.1	Matrix metalloproteinase 10 (stromelysin 2)	MMP10	–85.1	NM_002425.1
Secreted frizzled-related protein 4	SFRP4	12	NM_003014.2	FBJ murine osteosarcoma viral oncogene homologue B	FOSB	–74.1	NM_006732.1
Matrix metalloproteinase 26	MMP26	10.7	NM_021801.2	Interleukin 11	IL11	–60.3	NM_000641.1
Kynurenine 3-monooxygenase	KMO	10	BC005297.1	Serine (or cysteine) proteinase inhibitor, clade E	SERPINE1	–55	NM_000602.1
Polymyositis/scleroderma autoantigen 1	PMSCL1	10	AI346350	Chemokine (C-C motif) ligand 20	CCL20	–46.8	NM_004591.1
Gap junction protein (connexin-37)	GJA4	10.0	NM_002060.1	Interleukin 1, beta	IL1B	–45.7	NM_000576.1

C Gene name	Symbol	Stimulation	GenBank No.	**D** Gene name	Symbol	Inhibition	GenBank No.
Alkaline phosphatase, placental (Regan isozyme)	ALPP	11	NM_001632.2	Ribosomal protein S11	RPS11	–26.3	BF680255
Secretoglobin, family 1D, member 2	SCGB1D2	8.9	NM_006551.2	Ribosomal protein, large P2	RPLP2	–14.8	BC005354.1
Gelsolin (amyloidosis, Finnish type)	GSN	8.5	BE675337	Ribosomal protein L27	RPL27	–14.1	BE312027
Trefoil factor 1	TFF1	7.8	NM_003225.1	H3 histone, family 3A	H3F3A	–8.5	AA292281
GDNF family receptor alpha 2	GFRA2	7.6	U97145.1	Ribosomal protein L27a	RPL27A	–8.3	BE737027
Beta–1,3-glucuronyltransferase 3	B3GAT3	7.4	NM_012200.2	Ribosomal protein L14	RPL14	–7.9	BC000606.1
BCL2-like 1	BCL2L1	5.6	NM_001191.1	C-terminal binding protein 1	CTBP1	–7.1	AA053830
Triple-functional domain (PTPRF interacting)	TRIO	5.5	AF091395.1	glycine-N-acyltransferase	GLYAT	–7.1	AW024233
TUWD12	TUWD12	5.5	NM_172240	Ribosomal protein S19	RPS19	–6.2	BC000023.1
Lipopolysaccharide-binding protein	LBP	5.2	NM_004139.1	Calreticulin	CALR	–5.8	BE251303

E Gene	Symbol	Stimulation	GenBank No.	**F** Gene	Symbol	Inhibition	GenBank No.
Amylase, alpha 1A; salivary	AMY1A	4.3	NM_004038.1	Ribosomal protein L27a	RPL27A	–10.7	BE737027
Suppressor of cytokine signalling 1	SOCS1	2.8	AB005043.1	Ribosomal protein S20	RPS20	–6.5	AF113008.1
Haemoglobin, alpha 1	HBA1	2.6	NM_000558.2	Microtubule-associated protein, member 3	MAPRE3	–3.4	BG222594
Pregnancy-specific beta-1-glycoprotein 9	PSG9	2.2	NM_002784.1	NICE–1 protein	NICE–1	–2.5	NM_019060.1
Dipeptidylpeptidase 4	DPP4	2.2	M80536.1				
Metallothionein 1K	MT1K	2.1	R06655				
ATPase, H^+ transporting, V1 subunit A	ATP6V1A	2.1	NM_001690.1				
Eukaryotic translation initiation factor 2, subunit 3	EIF2S3	2.0	NM_001415.1				
Haemoglobin, beta	HBB	2.0	M25079.1				
Solute carrier family 30	SLC30A1	2.0	AI972416				

All data are presented as fold changes.

Table 4. Gene transcripts that responded similarly to 17β-E2 treatment in explant cultures of both M- and LATE-P-phase endometrium tissue.

Gene	Symbol	M phase	LP phase	GenBank no.
Ribosomal protein L27a	RPL27A	–8.3	–10.7	BE737027
Ribosomal protein S20	RPS20	–5.5	–6.5	AF113008.1
Orosomucoid 1	ORM1	2.1	1.7	NM_000607.1
Secretoglobin, family 2A, member 1	SCGB2A1	2.6	1.5	NM_002407.1
Ras-related associated with diabetes	RRAD	2.6	1.9	NM_004165.1
Chromosome 14 open reading frame 137	C14orf137	3.5	1.7	NM_023112.1
Lipopolysaccharide binding protein	LBP	5.2	1.6	NM_004139.1
Secretoglobin, family 1D, member 2	SCGB1D2	8.9	1.8	NM_006551.2

Data are presented as fold changes.

Table 5. Gene transcripts that were affected by 17β-E2 treatment in M-phase endometrium explants, and that were also increased or decreased in LATE-P-phase endometrium when compared to M-phase endometrium.

Gene	Symbol	Uncultured	Cultured	GenBank no.
Acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	ANP32A	2.0	3.0	NM_006305.1
Carboxypeptidase M	CPM	6.3	2.5	NM_001874.1
CD44 antigen (homing function and Indian blood group system)	CD44	–3.2	–2.0	AW851559
Cyclin A1	CCNA1	3.4	3.5	NM_003914.1
Dual specificity phosphatase 1	DUSP1	**–6.5**	**2.3**	AA530892
Eyes absent homologue 2 (*Drosophila*)	EYA2	4.7	3.0	U71207.1
Fibroblast growth factor 18	FGF18	–6.9	–2.2	BC006245.1
Fibroblast growth factor 9 (glia-activating factor)	FGF9	–3.6	–2.1	NM_002010.1
Gap junction protein, alpha 4, 37-kDa (connexin-37)	GJA4	10.0	3.5	NM_002060.1
Glycerol kinase	GK	**–5.8**	**2.1**	AI830490
GREB1 protein	GREB1	**2.6**	**–2.1**	AF245390.1
Histone 1, H2bh	HIST1H2BH	**–2.6**	**2.1**	NM_003524.1
Insulin-like growth factor-binding protein 3	IGFBP3	–4.1	–2.5	BF340228
Inter-alpha trypsin inhibitor heavy-chain precursor 5	ITIH5	8.9	2.5	NM_030569.1
Interferon-induced protein with tetratricopeptide repeats 1	IFIT1	**–2.6**	**2.6**	NM_001548.1
Keratin 7	KRT7	–2.4	–4.5	AI920979
Leukocyte-specific transcript 1	LST1	**–11.2**	**2.6**	AV713720
Leukocyte-derived arginine aminopeptidase	LRAP	3.2	3.6	NM_022350.1
Lysyl oxidase-like 2	LOXL2	**–6.9**	**2.4**	BE251211
Major histocompatibility complex, class II, DQ beta 1	HLA-DQB1	**–11.5**	**2.3**	M16276.1
Mitochondrial solute carrier protein	MSCP	**–2.3**	**2.6**	NM_018586.1
Natriuretic peptide receptor B/guanylate cyclase B	NPR2	4.6	2.3	NM_003995.2
Natural cytotoxicity-triggering receptor 3	NCR3	**–6.6**	**2.3**	AI735692
PiggyBac transposable element derived 5	PGBD5	2.6	2.5	NM_024554.1
Pituitary tumour-transforming 1	PTTG1	**3.9**	**–2.0**	NM_004219.2
Pituitary tumour-transforming 3	PTTG3	**2.7**	**–2.2**	NM_021000.1
Pleckstrin	PLEK	**–3.9**	**2.6**	AI433595
Progesterone receptor	PGR	9.1	3.0	NM_000926.1
Protein (peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1	PIN1	2.0	2.2	NM_006221.1
Prostaglandin-endoperoxide synthase 2	PTGS2	**–14.8**	**3.5**	NM_000963.1
Ras-related associated with diabetes	RRAD	**–4.0**	**2.6**	NM_004165.1
Ras-related GTP binding	D RRAGD	**–7.6**	**2.8**	AF272036.1
Ribosomal protein L37a	RPL37A	–2.6	–8.3	BE857772
S100 calcium-binding protein A8 (calgranulin A)	S100A8	–26.9	–2.1	AW238654
Secretoglobin, family 1D, member 2	SCGB1D2	4.9	8.9	NM_006551.2
Solute carrier family 15, member 3	SLC15A3	**–5.9**	**2.3**	NM_016582.1
SRY (sex-determining region Y)-box 4	SOX4	**–4.5**	**2.0**	AI989477
TUWD12	TUWD12	2.6	5.5	NM_003774.2

The bold text indicates genes that are oppositely regulated in vitro and in vivo. The expression of these genes is induced by 17β-E2 in vitro in M-phase endometrium, but reduced in LATE-P-phase endometrium when compared to M-phase endometrium, or reduced by 17β-E2 in vitro in M-phase endometrium, but induced in LATE-P-phase endometrium.

in this study justify our choices. Even though the menstrual endometrium was sometimes partly ischaemic, which impairs the tissue structure, we showed that the menstrual phase endometrium was superior with regard to oestrogen responsiveness (the number of differentially expressed transcripts being 200 vs 4 transcripts). In addition, when repeating the experiments using a selection of genes, most of the M-phase explants responded to 17β-E2. The fact that oestrogen receptor (ER) levels in M and LATE-P phase were similar [11] can therefore not explain the difference in responsiveness, indicating that other influences determine the high sensitivity of the menstrual endometrium. If an intact tissue structure was an important determinant in regulating gene expression, LATE-P-phase endometrium should be more responsive to oestradiol. However, this appeared not to be the case in this study.

A stringent scoring system was employed when filtering the array data, to minimise the occurrence of false-positive results, as a limited number of samples were used in the array hybridisations. The validity of this filtering system is confirmed by the finding that a number of genes known to be regulated by oestrogen were also revealed in the comparisons in this study (PR [17], TFF1/pS2 [18] and secreted frizzled-related protein 1 [19]). In addition, the responses found for the selected genes in the array comparisons, were verified using quantitative real-time PCR as an independent technique. The stringent scoring system could not account for all biological variation. In the additional uncultured endometrial tissues, the expression of selected genes did not always corroborate the array findings, which shows that the use of limited numbers of samples in the array hybridisations requires more elaborate validation. However, in the cultured biopsies, the findings in the independent experiments corroborated much better. Six of the seven tested genes also responded in the additional series of experiments. Apparently, the culture conditions alleviate some of the biological variability between biopsies.

Of the 794 transcripts differentially regulated when comparing uncultured endometrium tissue obtained from LATE-P phase and M phase, the majority were down-regulated (64%) in LATE-P-phase endometrium tissue. This may be due to the multiple processes that are ongoing during menstruation, i.e. tissue degeneration, inflammation, hypoxia, epithelial repair and angiogenesis. As expected, many inflammatory cytokines, enzymes involved in eicosanoid biosynthesis and immunomodulators and their receptors, as well as various angiogenic modulators were highly expressed in M-phase endometrium compared to LATE-P-phase endometrium. In addition, the destruction of the vasculature during menstruation creates a hypoxic environment which is illustrated by the elevated expression of hypoxia-induced proteins in M-phase endometrium, i.e. haem oxygenase-1 [20], adrenomedullin [21], carbonic anhydrase II [22], VEGF and hypoxia-inducible protein-2 [23].

The breakdown of the endometrium prior to and during menstruation involves MMPs [24]. In this study the expression of a number of MMPs was significantly higher in M-phase endometrium compared to LATE-P-phase endometrium. In contrast, MMP-26 (endometase) expression was higher in LATE-P-phase endometrium tissue. Endometase is an epithelial MMP, and is highly expressed around the period of implantation [25, 26]. This suggests that MMP-26 may not be involved in the endometrial breakdown process, but in endometrial remodelling prior to and during trophoblast invasion.

The wound-healing process after menstruation begins with the re-epithelialisation of the uterine surface [27]. This process is complete on day 5 or 6 of the menstrual cycle. In the absence of oestrogen (i.e. after ovariectomy), the endometrium has the capacity to end endometrial bleeding and to begin the healing process, indicating that other factors are involved. Factors that have been implicated in epithelial cell repair are the trefoil peptides. Three trefoil peptides, TFF1/pS2, TFF2/SP and TFF3/ITF were found to be stimulated by 17β-E2 in M-phase endometrium. The trefoil family of peptides consists of mucin-associated peptides found predominantly in mucus-secreting cells of the gastrointestinal mucosa [28] and have been implicated in the regeneration of the mucosal surface after damage [29]. The trefoil factors possibly play a similar role in the regeneration of the endometrium.

Oestrogen responsiveness was also demonstrated for three members of the uteroglobin family: secretoglobin 1D2 (lipophilin B), secretoglobin 2A1 (mammoglobin B) and secretoglobin 2A2 (mammoglobin). Mammaglobins were first identified in glandular epithelium of the breast [30], and were later also described in human endometrium [31]. This study showed that this family of genes is regulated by oestrogen. Mammaglobin B forms covalent heteroduplexes with lipophilin B [32] and this complex was found to be the biologically relevant form of mammaglobin. No detailed information is available with regard to the functions of lipophilin B and the mammaglobins. However, uteroglobin or lipophilin A was shown to inhibit tumour [32] and trophoblast invasion, to inhibit tumour angiogenesis and transform normal tissue into a neoplastic phenotype [33]. In other words, the secretoglobins may transform the proliferating cells into a differentiated state. Furthermore, roles as immunoregulators and anti-inflammatory proteins have also been suggested [34].

Analogous to the reduced expression of the majority of genes in LATE-P-phase endometrium compared to M-phase endometrium, the responsiveness of LATE-P-phase endometrium to 17β-E2 in vitro was also much lower compared to that of M-phase endometrium (16 vs

193 genes). The reduced responsiveness of the LATE-P-phase endometrium is in line with the supposition that prolonged exposure to oestrogen desensitises the endometrium [35, 36]. One is tempted to suggest that this may be an intrinsic mechanism that maintains the endometrium in a proper state of differentiation. This is further supported by the findings Younis et al. of [37, 38] and others [39] who demonstrated that oestrogen exposure for less than 6 days is not sufficient to support successful implantation, whereas after prolonged exposure to oestrogen (35 days), the endometrium is still responsive to progesterone and is receptive.

Two groups of genes that were shown to be involved in the morphogenesis of the urogenital tract and adult uteri and that were shown to be differentially expressed in uncultured LATE-P-phase endometrium tissue when compared to uncultured M-phase endometrium tissue are mediators of Wnt signalling and the homeobox-containing genes. Expression of Wnt5a and Wnt7a genes was lower in LATE-P-phase endometrium, whereas the expression of secreted frizzled-related proteins 1 and 4 (sFRP) and Wnt inhibitory factor-1 (WIF-1), which are known Wnt antagonists [40], was significantly higher in LATE-P-phase endometrium. The homeobox-containing proteins Hoxa10, Hoxa11 and Hoxb7 were all increased in LATE-P-phase compared to M-phase endometria.

Wnt5a is predominantly expressed in stroma, whereas Wnt7a is mostly expressed in luminal epithelium [41, 42] and they play crucial roles in uterine gland development in the mouse. As these genes are also expressed in a similar manner in human endometrium [43], Wnt5a and Wnt7a are likely also involved in gland development in the human endometrium. Carta and Sassoon [44] demonstrated that exposure to diethylstilbestrol (DES), an oestrogen agonist, reduces Wnt7a expression in the murine uterus, and this response is dependent on the presence of Wnt5a [42].

The expression of Hoxa10 and Hoxa11 genes is down-regulated by DES in normal mice [45], but not in Wnt5a and Wnt7a –/– mice [42, 44]. Low oestrogen levels during the menstrual period may allow expression of Wnt5a and Wnt7a, which initiate gland development, and increasing levels of circulating oestrogens could suppress the expression of Wnt5a and Wnt7a to allow expression of Hoxa10 and Hoxa11, which complete the gland development and differentiation process. However, even though DES is an potent oestrogen agonist, its effects are not always similar to that of oestradiol [45, 46]. These findings do illustrate, however, the importance of concerted actions between the stroma (Wnt5a) and epithelium (Wnt7a) in the regulation of endometrial development.

Surprisingly, we also observed up-regulation of several inhibitors of Wnt signalling, sFRP1, sFRP4 and WIF-1, in LATE-P-phase endometrium. Their significance is not known; however, they may protect the silencing of Wnt signalling to allow the expression of Hoxa10 and Hoxa11, promoting endometrium differentiation. Recently, evidence was provided that sFRP1 is also directly involved in vascular remodelling and maturation. sFRP1 expression was highest in vascular endothelium [47], expression could be stimulated by oestrogens [19], and sFRP1 induces larger, longer vessels and is apparently associated with more pericytes compared with vessels formed under control conditions [48]. sFRP4 expression is particularly high in proliferating stroma [49] and may be a paracrine factor involved in the stimulation of endometrial growth.

The gene coding for the homeobox-containing protein Hoxb7, also shown to be an inducer of vascular development [50], was up-regulated in LATE-P-phase compared to M-phase endometria as well. The elevated expression in LATE-P-phase endometrium suggests a role for Hoxb7 in vascular development under normoxic conditions.

With regard to the differentiative actions of oestrogen in the human endometrium, of interest is that the expression of connexin-37 is up-regulated by 17β-E2 in M-phase endometrial explants. Connexin-37 is a gap junction protein expressed in endometrium, predominantly in endothelial cells [51]. In connexin-37 knockout mice, early vasculogenesis proceeds normally, but by the age of 18.5 days, animals display abnormal vascular channels with localized haemorrhages in various organs [52, 53]. As a result of these developmental defects, the mice lack mature Graaffian follicles, fail to ovulate and develop numerous inappropriate corpora lutea [54]. Connexin-37 is therefore likely to play a role in the oestrogen-dependent regeneration of the vasculature after menstruation, and is thus involved in the development of the endometrium**.**

In summary, we have shown that the role of 17β-E2 in the repair and development of the endometrium during the proliferative phase of the menstrual cycle is certainly not limited to just the regulation of proliferation. This is illustrated by the differential expression of genes involved in processes such as blood vessel growth and maturation, and morphogenesis of the endometrium in the late proliferative phase. Some genes are regulated directly by 17β-E2, whereas the expression of others is modulated by the changing uterine environment. This study emphasises the importance of oestrogen with regard to endometrium maturation and differentiation.

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