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Changes in gene expression during the early to mid-luteal (receptive phase) transition in human endometrium detected by high-density microarray screening

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High density cDNA microarray screening was used to determine changes in gene expression occurring during the transition between the early luteal (prereceptive) and mid-luteal (receptive) phases in human endometrium. Of ~12 000 genes profiled, 693 (5.8%) displayed >2-fold differences in relative levels of expression between these stages. Of these, 370 genes (3.1%) displayed decreases ranging from 2- to >100-fold while 323 genes (2.7%) displayed increases ranging from 2- to >45-fold. Many genes correspond to mRNAs encoding proteins previously shown to change in a similar manner between the proliferative and mid-luteal phases, serving as one validation of the microarray screening results. In addition, novel genes were identified. Genes encoding cell surface receptors, adhesion and extracellular matrix proteins and growth factors accounted for 20% of the changes. Several genes were studied further by Northern blot analyses. These results confirmed that claudin-4/*Clostridium perfringens* enterotoxin (CPE) receptor and osteopontin (OPN) mRNA increased ~4- and 12-fold respectively, while betaig-H3 (BIGH3) decreased >80% during the early to mid-luteal transition. Immunostaining also revealed strong specific staining for claudin-4/CPE, EP_1 and prostaglandin receptor in epithelia, and leukotriene B4 receptor in both epithelia and stroma, at the mid-luteal stage. Collectively, these studies identify multiple new candidate markers that may be used to predict the receptive phase in humans. Some of these gene products, e.g. OPN, may play direct roles in embryo–uterine interactions during the implantation process.

Key words: endometrium/gene expression/implantation/microarray/uterine receptivity

Introduction

Embryo implantation is critically dependent on the arrival of an attachment-competent blastocyst in co-ordination with generation of a receptive uterine state. The acquisition of attachment competence by the embryo follows hatching from the zona pellucida and is accompanied by expression of cell adhesion-promoting molecules on the external trophectodermal surface (Carson *et al.*, 2000). Uterine receptivity is regulated by the actions of ovarian steroid hormones produced by the corpus luteum (Psychoyos, 1986). In humans, the period of receptivity is believed to occur ~7–9 days post-ovulation during the mid-secretory or mid-luteal phase (Lessey and Castelbaum, 1998; Wilcox *et al.*, 1999). In response to ovarian steroids, characteristic morphological changes occur in the endometrium, and these are responses routinely used to stage the human endometrium (Noyes *et al*., 1950). Such staging is particularly important as inappropriate or delayed development of the endometrium may cause miscarriage or infertility (Wilcox *et al.*, 1999). Inappropriate endometrial development during IVF/embryo transfer protocols may account for the relatively low individual pregnancy success worldwide (Report ASRM/SARTR, 2000). These observations suggest that additional indicators are needed to detect underlying endometrial defects that may make individuals poor candidates for such procedures.

The best markers of a true receptive state would be those molecules directly involved in the initial stages of the implantation process; however, this necessitates functional testing of these marker molecules, an impractical approach in humans. Such testing can be performed in animal models and several markers of the receptive state have been proposed based on correlative studies in humans as well as a combination of correlative and functional studies in animals (Carson *et al.*, 2000; for review). In most cases, identification of these markers was the result of careful examination of the expression of particular proteins or mRNAs based on preconceived ideas or serendipitous observations. The recent advent of high throughput microarray screening for the expression of human genes has allowed an unbiased approach toward identifying changes in gene expression that accompany transition of the human endometrium from a prereceptive (early luteal) to a receptive (mid-luteal) state. Using an existing microarray display comprising of 12 000 genes (~25–33% of the human genome) (Clavrie, 2001) we identified a subset of genes that are altered during this transition. A few of these genes correspond to previously suggested markers, while others represent novel potential markers of

human receptivity. These studies provide a basis for performing more detailed studies on the expression and function of these novel markers in both human and animal implantation models.

Materials and methods

Human subjects and tissue staging

Endometrial samples were obtained from a larger pool of fertile volunteers. Subjects monitored their urinary LH excretion daily from cycle day 10 using a commercial ovulation predictor kit (Ovuquik One-Step; Quidel, San Diego, CA, USA) and were instructed to contact the study co-ordinator on the day that the mid-cycle LH surge was first detected (luteal day 0). At time of enrolment, subjects were randomized to undergo endometrial sampling on a specific day defined by a randomization schedule (luteal day 1–14). All returned on the appointed day for endometrial biopsy that was performed using an endometrial pipelle. From a pool of tissue specimens we selected three from the early secretory phase (LH day 2–4) and three from the mid-secretory phase (LH day 7–9). Each subject was sampled in a single menstrual cycle, and all selected samples had histological changes consistent with their LH day. Blood samples for measurement of serum progesterone were obtained on the day of endometrial sampling, and in each case ovulation was confirmed on the basis of an elevated serum progesterone. Endometrial tissues were snap-frozen in liquid nitrogen immediately after collection and a portion of each specimen was fixed in 10% (w/v) buffered formalin before paraffin-embedding for histological examination and dating. The use of human subjects and the procedures were approved by the Institutional Review Boards of both the University of North Carolina and the University of Delaware.

Northern blot analysis

Total RNA was extracted from frozen human endometrial tissue samples using Trizol Reagent according to the manufacturer's instructions (Life Technologies, Gaithersburg, MD, USA). Quantitation and estimation of purity were performed by measuring absorbance of each RNA sample at UV wavelengths of 260 and 280 nm, and integrity was determined by visual inspection of RNA fractionated by agarose gel electrophoresis. Northern blot analysis was performed using the NorthernMax-Gly kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. Briefly, 9 μg of glyoxylated total RNA from each sample were separated on 1% (w/v) agarose gels and downward-transferred to BrightStar-Plus positively-charged nylon membrane (Ambion). After transfer, RNA was crosslinked to the membrane by exposure to ultraviolet light. Prehybridization and hybridization were carried out at 42°C in UltraHyb buffer (Ambion). The cDNA probe used for detection of claudin-4 mRNA was a 470 bp RT–PCR product, confirmed by sequencing, made from late luteal phase endometrial RNA. Primers were designed to amplify a segment of claudin-4 (GenBank accession no.: NM_001305.1). Respective forward and reverse primers used for PCR amplification of claudin-4 cDNA were as follows: 5′-CCGCACAGACAAGCCTTACT-3′ and 5′-TACCCGGAACAGAGGAGATG-3′. Primers were also designed to amplify a segment of human OPN cDNA (GenBank accession no.: NM_000582) and found to be unique by a BLAST search. Respective forward and reverse primers were 5′-TTGCTTTTGCCTCCTAGGCA-3′ and 5′-GTGAAAATT-CGGTTGCTGG-3′. Forward and reverse primers for BIGH3 cDNA (GenBank accession no.: M77349) were 5′-TCTCCCGTGACTTTCGTCTT-3′ and 5′-CCAGCAGCAATCTTTTAGCC-3′ respectively. All primers were designed to amplify unique sequences in each case as determined by a BLAST search. Oligonucleotides were purchased from Sigma Genosys (St Louis, MO, USA). A cDNA probe for human 18S ribosomal RNA (American Type Culture Collection, Manassas, VA, USA) was used as a load control. Probes were non-isotopically labelled using the BrightStar Psoralen-Biotin Nonisotopic Labelling Kit (Ambion). Hybridization signals were detected using the BrightStar BioDetect Kit (Ambion). Blots were exposed to X-ray film, and bands were quantified using the NIH/Scion Image Beta 3b program (available online at http://www.scioncorp.com). Blots were stripped between probings with boiling 0.1% (w/v) sodium dodecyl sulphate in water, then stored at 4°C. Northern blots were performed twice on two separate series of samples with similar results.

Microarray screening

Total RNA was extracted from the frozen human endometrial tissue samples using Trizol reagent (Life Technologies). RNA was then purified by the Rneasy kit (Qiagen, Valencia, CA, USA) and used to probe the Human Genome U95A Array (Affymetrix, Sunnyvale, CA, USA). For microarray screening, 7 μg of total RNA was used to synthesize cDNA. A custom cDNA kit from Life Technologies was used with a T7-$(dT)_{24}$ primer for this reaction. Biotinylated cRNA was then generated from the cDNA reaction using the BioArray High Yield RNA Transcript Kit. The cRNA was fragmented in fragmentation buffer (5× stock: 200 mmol/l Tris-acetate, pH 8.1, 500 mmol/l KOAc, 150 mmol/l MgOAc) at 94°C for 35 min before chip hybridization. A total of 15 μg of fragmented cRNA was then added to a hybridization cocktail [0.05 μg/μl fragmented cRNA, 50 pmol/l control oligonucleotide B2, *BioB*, *BioC*, *BioD*, and *cre* hybridization controls, 0.1 mg/ml herring sperm DNA, 0.5 mg/ml acetylated bovine serum albumin, 100 mmol/l MES, 1 mol/l Na^+, 20 mmol/l EDTA, 0.01% (v/v) Tween 20]. Arrays were hybridized for 16 h in the GeneChip Fluidics Station 400 and then washed and scanned with the Hewlett Packard GeneArray Scanner. Affymetrix GeneChip Microarray Suite 4.0 software was used for washing, scanning and basic analysis. Detailed information on the gene array system is available at www.affymetrix.com. The samples were analysed in duplicate and sample quality was assessed by examination of 3′ to 5′ intensity ratios of certain genes. GeneSpring software (version 4.0.2; Silicon Genetics) was used to organize and statistically analyse the results.

Immunostaining

Frozen mid-luteal human endometrial sections (8 μm) were fixed for 10 min at room temperature in methanol and rehydrated in phosphate-buffered saline (PBS) for 10 min with one change of buffer. Sections were then incubated at 37°C with EP_1 polyclonal receptor antibody (Cayman, Ann. Arbor, MI, USA) diluted to 20 ng/μl with PBS or leukotriene B_4 polyclonal receptor antibody (Cayman) diluted to 4 ng/μl in PBS for 1 h. Non-immune rabbit IgG (Zymed) was used as a negative control for each antibody. Sections were rinsed three times for 5 min in PBS at room temperature and incubated with fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Amersham, Piscataway, NJ, USA) diluted 1:10 in PBS at 37°C for 40 min. Following three additional 5 min rinses in PBS, samples were mounted in glycerol:PBS containing 0.01% (w/v) *p*-phenylenediamine to prevent fading and photographed using an epifluorescence microscope. At least two samples from separate patients were stained at each stage.

The anti-claudin-4 antibody (3E2C1; Zymed) is a monoclonal mouse antibody, raised against the synthetic peptide corresponding to a 22 aa sequence at the C-terminus of human claudin-4 protein. Paraffin-embedded sections (5 mm) were deparaffinized in xylene and rehydrated in ethanol with increasing concentrations of water. Endogenous peroxidase activity was quenched with 3% (v/v) hydrogen peroxide for 30 min. Non-specific binding sites were blocked with 2% (v/v) normal goat serum (NGS) for 30 min at room temperature. Primarily antibody was serially diluted in a solution of PBS containing 1% (v/v) NGS and 0.1% (w/v) sodium azide to optimize the appropriate concentrations in order to achieve maximum sensitivity and specificity. Tissue sections were incubated with primary antibody at 4°C overnight at 1:1000 dilution. Negative control sections were treated with non-immune serum diluted in the same manner. After primary antibody incubation, sections were washed twice with PBS followed by treatment with 1% (v/v) NGS for an additional 30 min. Subsequently, sections were washed with PBS and incubated with biotinylated goat anti-mouse secondary antibody (Vectastain Elite ABC Kit; Vector Laboratories, Inc., Burlingame, CA, USA) at a dilution of 1:400 for 30 min at room temperature. After rinsing with PBS, the immunoreactive antigen was visualized using avidin–biotin peroxidase complex (Vectastain Elite ABC kit) and 3,3′-diaminobenzidine as chromagen. Slides were counterstained with Mayer's haematoxylin blue/Toulidine Blue followed by dehydration in a graded series of ethanols, cleared in xylene, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ, USA). The resulting stain was evaluated on a Nikon microscope by a single blinded observer.

Results

Microarray screening

Total endometrial RNA from six fertile women was extracted and pooled from the early luteal ($n = 3$) and mid-luteal ($n = 3$) phases.

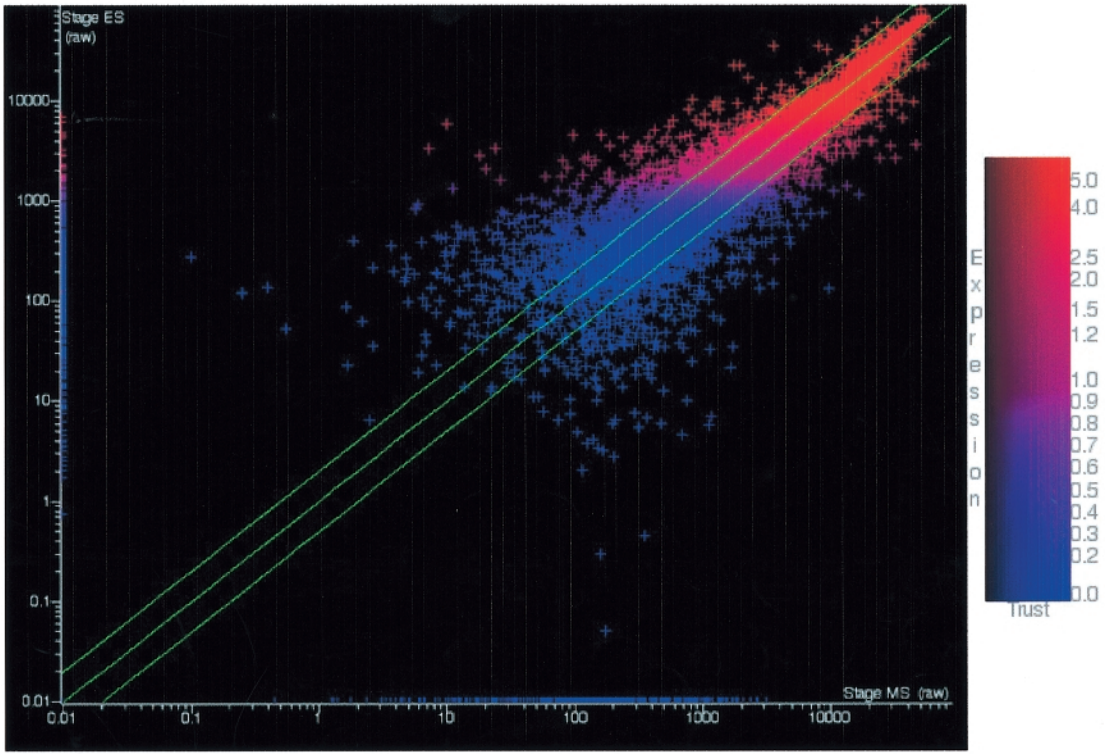

Figure 1. Scattergram of changes in gene expression between early and mid-secretory phases in normal women. The graph shows the comparison of the relative expression of each of the 12 000 genes in the mid-secretory (MS; *x*-axis) versus the early secretory (ES; *y*-axis) phase from a representative analysis. The three diagonal lines represent the mean and upper and lower 2-fold difference boundaries.

These RNA preparations were then used to probe the U95 human gene microarray comprising of ~12 000 genes. The results of this screening are compiled in the scattergram shown in Figure 1. The vast majority (94.2%) of the genes represented displayed <2-fold changes between early and mid-luteal phases. These included many genes previously shown to display no or modest changes during the menstrual cycle. A list of all genes that were noted to increase or decrease by ⩾2-fold can be viewed as supplementary data online (www.molehr.oupjournals.org).

A total of 323 genes displayed significant increases in expression of ⩾2-fold between the early luteal and mid-luteal phase. Cell surface proteins, extracellular matrix components and growth factors/cytokines were the major group of genes of known function altered under these conditions and accounted for 23% of the total (Tables I and II). DNA binding proteins, transcription factors and DNA modifying enzymes accounted for 8% of these genes (Tables I and III), intracellular signalling/cell cycle proteins, metabolic enzymes, genes encoding proteins of miscellaneous other functions and proteins of unknown function or expressed sequence tagges (ESTs) accounted for 12, 17, 7 and 30% of the genes with increased expression respectively (Table I; http://www.med.unc.edu/obgyn/tables/increase.htm).

A similar number of genes (370) were identified as displaying decreases ⩾50% during the early to mid-luteal phase transition. Again, genes encoding cell surface proteins, extracellular matrix components and growth factors/cytokines were the major group of genes of known function altered and constituted 19% of the total (Tables I and IV). Transcription factors and DNA modifying enzymes accounted for 13% of this list (Tables I and V). A number of genes associated with cell cycle progression were down-regulated during this transition including p55CDC, cyclin B, CD22 and cdk3. These, along with intracellular signalling proteins, constituted 10% of the decreases while metabolic enzymes, proteins of miscellaneous other functions and proteins of unknown function or ESTs accounted for 12, 11 and 35% of the changes respectively (Table I; www.med.unc.edu/obgyn/tables/decrease.htm).

Table I. Distribution of changes in gene expression among different functional classes[a]

Functional class	% of subset that increase	% of subset that decrease
Cell surface proteins, extracellular matrix components, and growth factors/cytokines	23	19
Transcription factors and DNA modifying enzymes	8	13
Metabolic enzymes	17	12
Intracellular signalling and cell cycle proteins	12	10
Genes of other known functions	7	11
Genes of unknown functions and ESTs	33	35

[a]Expression of a total of 323 and 370 genes which were increased and decreased, respectively, by a factor of ⩾2-fold, between early and mid-luteal phases.

From these results, it was evident that genes encoding many different types of proteins were significantly altered in expression during the early to mid-luteal transition. Nonetheless, cell surface, extracellular matrix components and growth factors/cytokines constituted a large proportion of the overall changes (20%). Verification of these observations relied on several criteria: (i) statistical analyses of changes in the relative expression of particular genes in RNA from multiple, fertile women; (ii) detection of similar changes in relative expression of the same gene at independent sites on the microarray chip; and (iii) detection of previously reported changes in gene

Table II. Relative expression of genes encoding cell surface components, extracellular matrix components and growth factors/cytokines that increase between the early and mid-luteal phases[a]

Gene	Accession number	Relative expression	Gene	Accession number	Relative expression
Activinβ-C	X82540	2.0	Prostatic acid Phosphatase/PAP	M24902	2.9
GABA-R	Y09765	2.0	NKG5	M85276	2.9
DRASIC/TNAC1	AB010575	2.0	ENaCa/SCCN	X76180	3.0
Γ-glutamyl transpeptidase	J04131	2.0	Butyrophilin-like R3/BTNLR	AB020625	3.0
VLDL-R	D16532	2.0	DRM/GREMLIN	AF045800	3.0
Integrin α_{10}	AF074015	2.0	MCSP/melanoma-associated proteoglycan-3	X96753	3.0
Nicotinic cholinergic R β2	U62437	2.0	Apolipoprotein D	J02611	3.1
CGM1/CD66d	L00693	2.1	BMP R1B/ALK6	U89326	3.1
Fc-ε-R γ-chain	M33195	2.1	GABAA R γ3	S82769	3.2
Trail-R3	AF014794	2.1	C-C chemokine R type1	L09230	3.4
COMP	M55683	2.1	Jagged 1	AF003837	3.4
Dystrobrevinβ/BDTN	AF022728	2.2	NKp46	AJ006123	3.4
Desert hedgehog	U59748	2.2	EVA1	AF030455	3.6
T-cell R δ	M21624	2.2	FLT1	S77812	3.6
IL15	U14407	2.2	Ca channel α_{14}	AF051946	3.6
NPY4R	U42387	2.2	Ca-activated K channel	U02632	3.8
B7-2/CD28	U04343	2.3	Neurexin3	AB018286	3.9
Corticostatin	AF013252	2.3	Apolipoprotein L	AF019225	3.9
Sperm-specific antigen-2	M61199	2.3	Vgf	Y12661	4.1
Cadherin-1	Z35402	2.4	Edg6	AJ000479	4.3
LIR/CMRF35	X66171	2.4	Cystatin F	AF031824	4.5
MUC8	U14383	2.5	GPR31	U65402	5.2
Insulin R	M10051	2.5	Involucrin	M13903	5.5
Fibulin2	X82494	2.5	Prostaglandin E R1/EP1	L22647	5.7
A33	U79725	2.6	Interferon α R2	L42243	5.7
ZO1	L14837	2.6	TFPI-2/PP5	D29992	6.3
GDF15/MIC1	AB000584	2.6	Acetylcholine Rβ	X14830	6.4
Renin	M26901	2.6	Leukotriene B4 R	D89078	9.2
NCCT	X91220	2.6	Mammoglobin1/UGB2	U33147	10.6
Ectonucleosidetriphoshate diphosphohydrolase/CD39L1	U91510	2.7	Osteopontin	J04765	12.3
Ninjurin1	U91512	2.8	Dickkopf1/DKK1	AB020315	12.6
Testican	X73608	2.8	Claudin4/CPE R	AB000712	45
PALB/Transthyretin	D00096	2.8	Wnt10B	U81787	>45
GLU R5	L19058	2.8	Integrin α_9	D25303	>45
LIR4	AF025527	2.9	Integrin β_7	M68892	>45

[a]Data are expressed as ratios of mid-luteal:early luteal values for each gene. In all cases, $P < 0.05$. R = receptor. Other abbreviations represent standard nomenclature for the respective genes.

Table III. Relative expression of genes encoding transcription factors and DNA modifying enzymes that increase between the early and mid-luteal phases[a]

Gene	Accession number	Relative expression	Gene	Accession number	Relative expression
COUP-TF	X16155	2.0	Mineralocorticoid R	M16801	2.3
PAX2	M89470	2.0	TR3 orphan R	L13740	2.3
Homeobox Og12	AF023203	2.0	XRCC9/FANCG	AC004472	2.4
HOX5.1/HOX4B	X17360	2.0	EBBP	H06628	2.5
PPARδ/NUC1	L07592	2.0	JunB	X51345	2.5
HNF2/HNF1β	X58840	2.1	H2A/d	Z98744	2.5
ER81	U17163	2.1	NOT/NURR1	X75918	2.7
H2B/g	Z80779	2.2	NF1	U18760	2.7
SUPT6H	U46691	2.2	RAD51/XRCC2	Y08837	2.8
RNA polymerase 1	AF001549	2.2	OPTX2/SIX9	AJ011785	2.9
TIP30	AF039103	2.3	HOX11-like1	AJ002607	2.9
TAFII 170	AF038362	2.3	SF-1	U76388	4.2
XPAC	U16815	2.3	AP-2γ	U85658	4.7

[a]Data are expressed as ratios of mid-luteal:early luteal values for each gene. In all cases, $P < 0.05$. R = receptor. Other abbreviations represent standard nomenclature for the respective genes.

expression. In the latter case, similar changes in fibroblast growth factor (FGF) pathway components (Sangha *et al.*, 1997), PP5 (Butzow *et al.*, 1986) decay accelerating factor/CD55 (Kaul *et al.*, 1996), matrix metalloproteinase (MMP)-7/matrilysin (Rodgers *et al.*, 1993) and cyclin B (Shiozawa *et al.*, 1996) have been reported. Nonetheless, there were many examples of genes displaying large changes that

Table IV. Relative expression of genes encoding cell surface components, extracellular matrix components and growth factors/cytokines that decrease between the early and mid-luteal phases[a]

Gene	Accession number	Relative expression	Gene	Accession number	Relative expression
Neuromedin U	X76029	0.50	CD1e	X14975	0.38
Integrin α_{IIb}/CD41B	M34480	0.49	TYRO3	U02566	0.38
HB-1	AF103884	0.49	TIMP2	M32304	0.37
IL3 Rα/IL3RAY	D49410	0.49	NCAM1/CD56	S71824	0.37
AG2/HAG2	AF038451	0.48	Thyrotrophin releasing hormone	M63582	0.37
Aminopeptidase/DPPX	M96859	0.48	BMP10	AF101441	0.35
PSGL1	U25956	0.48	Neogenin	U61262	0.35
Secretagogin	Y16752	0.48	FGF9	D14838	0.35
GABA B R1	AL031983	0.48	NPYR Y2	U50146	0.35
Ephrin A2	AJ007292	0.48	Slit1	AB017167	0.34
Autotaxin	L35594	0.48	Ca channel α_{1D}	M76558	0.34
Integrin α_5	X06256	0.47	$CD1/CD1_C$	M28827	0.33
Collagen α2 (XI)	U41068	0.47	Ephrin B1/EPL62	U09303	0.31
MEGF7	AB011540	0.47	PTP-U2	U20489	0.31
LRP6	AF074264	0.47	Integrin β_2	M15395	0.31
Lumican	U21128	0.46	FGF R4	L03840	0.29
APLP1	U48437	0.46	Prolactin R	M31661	0.29
KCNJ14-PEN (K channel)	Y10745	0.46	OLF3	AC004853	0.29
TRAIL R2/DR5	AF016266	0.45	NPY3 R/fusin	L06797	0.27
PAR4	AF055917	0.45	TP120/CD6	U34624	0.26
Flotillin2	M60922	0.45	NMDA R1	L13266	0.25
PSA/KLK3	X07730	0.44	Uromodulin	M15881	0.23
FGF R1	X66945	0.44	Stromal cell-derived factor-1	L36033	0.21
GABA A R1δ	AF016917	0.44	Endothelin-3	X52001	0.20
Nidogen 2	D86425	0.43	Indian hedgehog	L38517	0.19
Fibroblast activation protein/FAPA	U09278	0.43	H2A.X	X14850	0.14
Ia-associated g chain	M13560	0.43	Dopamine R2	X51362	0.14
ALK1	Z22533	0.43	LDL R-related protein-3 LRp105	AB009462	0.14
Butyrophilin (BTF4)	U90546	0.43	MMP-11/stromelysin-3	X57766	0.10
Toll-like R6	AB020807	0.43	FrpHE	AF026692	0.09
MCP-3	X72308	0.42	BIGH3/betaig-H3	M77349	0.08
HLA-DPA2	M11591	0.42	Connexin 37	M96789	0.05
Protocadherin 68 (PCH68)	AF029343	0.42	Proenkephalin	J00123	0.04
TGFβ3	M60556	0.41	MMP-7/matrilysin	L22524	<0.01
Eotaxin	U46573	0.41	Fas	X89101	<0.01
Collagen VII α1	L02870	0.39	TRAP3/TNF R-associated factor-2[a]	U12597	<0.01

Data are expressed as ratios of mid-luteal:early luteal values for each gene. In all cases, $P < 0.05$. R = receptor. Other abbreviations represent standard nomenclature for the respective genes.

had not been reported previously. We performed Northern blots for a select group of genes (of known functions) that displayed large changes to further confirm the results of the microarray analyses.

Northern blot analyses confirms changes detected by microarray analyses

Several genes displaying >4-fold changes during the early to mid-luteal transition were chosen for verification by Northern blot analyses (Figures 2 and 3). Claudin-4/*Clostridium perfringens* enterotoxin (CPE) receptor (Kaul *et al.*, 1996) showed a large (4-fold) increase in expression during the early to mid-luteal transition (Figure 3), although the increase was not as large as predicted by the microarray results. Osteopontin (OPN) displayed a very large increase (>10-fold) in both the microarray and Northern analyses (Figure 2). More detailed studies have been performed recently revealing similar changes in OPN expression associated with uterine epithelium (Apparao *et al.*, 2001). Betaig-H3 (BIGH3), a transforming growth factor β-induced extracellular matrix protein with cell adhesion-promoting activity (Kim *et al.*, 2000), decreased by 92% by the microarray analysis and >80% by Northern blot analysis (Figure 3).

Immunostaining confirms marker protein expression detected by microarray analyses

Antibodies to several proteins encoded by genes displaying ⩾4-fold changes during the early to mid-luteal transition were used to stain mid-luteal stage uterine tissues. Prostaglandin receptor EP_1 was strongly expressed in epithelial cells closely associated with cell boundaries (Figure 4A). In contrast, leukotriene B_4 receptor was found in both epithelial and stromal cells in a pattern suggesting nuclear association (Figure 4B). Non-immune rabbit IgG did not stain any of these structures (Figure 4C). Antibodies to claudin-4/CPE receptor strongly stained both lumenal and glandular epithelia, but not stroma (Figure 5B). Collectively, these studies confirmed several of the results of mRNA expression by demonstrating protein accumulation in particular uterine cell types during the receptive phase.

Discussion

Recent technological advances have aided the practice of reproductive medicine in the last 15 years (Wilcox *et al.*, 1999). Much of this success can be attributed to improvements in embryo selection and

Table V. Relative expression of genes encoding transcription factors and DNA modifying enzymes that decrease between the early and mid-luteal phases[a]

Gene	Accession number	Relative expression	Gene	Accession number	Relative expression
MAZ	M94046	0.50	GCFX/PHOG	U89331	0.42
DNA primase	X74331	0.49	PCNA	M15796	0.41
PE-2/ERF	U15655	0.49	Transcriptional activator of c-fos promoter	U49857	0.40
Forkhead-like 7	AF078096	0.49	Dp-1	L23959	0.39
MMSET/TRX5	AJ007042	0.48	p300	U01877	0.37
MID1	AF041210	0.48	CAF1	U20980	0.37
STAT1	M97936	0.48	Histone deacetylase 6	AJ011972	0.36
HNF3β	AB028021	0.47	Snm-1	D42045	0.35
Staf50	X82200	0.47	HCAP-H	D38553	0.34
MSX-2	D89377	0.47	Nucleoporin	U43279	0.33
ERT	AF017307	0.46	MLH1/HNPCC	AF001359	0.32
HOXA9/HOX1G	U82759	0.46	TOPO2	J04088	0.31
Nucleolar protein 3	AF043244	0.46	CDX2	U51096	0.29
HMMH/OGG1	Y11731	0.46	CAPC	AB019987	0.29
XRCC3	AF035586	0.45	OCT-6	L26494	0.28
C/EBPα	Y11525	0.45	HOX 1.3/HOX1C	M26679	0.26
RAG2	AF080577	0.45	RCC1	D00591	0.24
DBP	U48213	0.45	Nucleoporin 24/CAIN	X64228	0.21
Chimeric DNA-binding factor	M29204	0.44	DNAse III	AJ243797	0.21
NF-AT3	L41066	0.44	Centromere protein A	U14518	0.20
TBX2	U28049	0.43	Skn-1a/PLA-1	AJ012214	0.19
HFH-4/HNF-3	X99350	0.43	ERR-α/ERR1	L38487	0.17
TAF-II 130	U75308	0.42	BAF60C	U66619	0.12
DNA ligase III/LIGIII	X84740	0.42			

[a]Data are expressed as ratios of mid-luteal:early luteal values for each gene. In all cases, $P < 0.05$. R = receptor. Other abbreviations represent standard nomenclature for the respective genes.

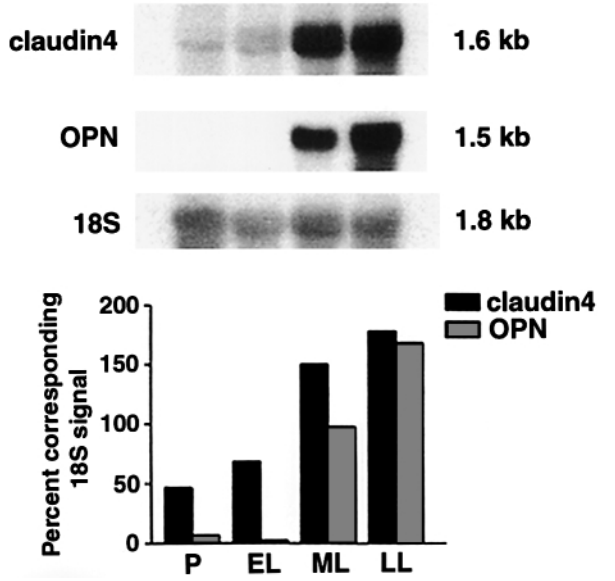

Figure 2. Northern blot analyses of changes in claudin-4/CPE receptor and osteopontin (OPN) expression. The top three panels show the Northern blots probed with cDNA to claudin-4/CPE receptor, OPN and 18S rRNA (18S) as a transfer efficiency and load control. The four stages of the menstrual cycle examined were (left to right): proliferative (P), early luteal (EL), mid-luteal (ML) and late luteal (LL). The graph at the bottom summarizes these data normalized to the 18S signal with the grey and black bars representing the relative changes in OPN and claudin-4 signals respectively.

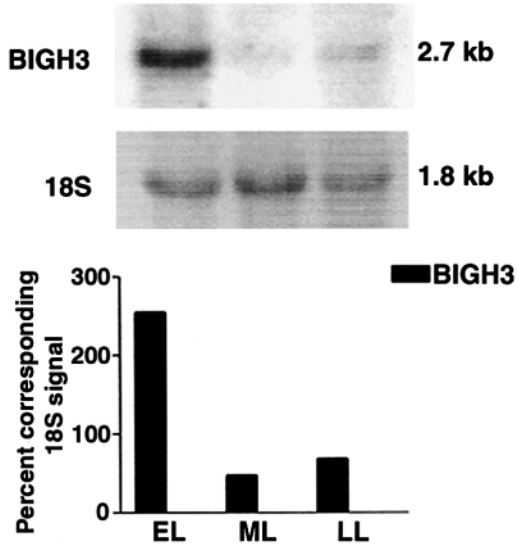

Figure 3. Northern blot analysis of betaig-H3 (BIGH3) expression. The top two panels show Northern blots probed with cDNA to BIGH3 and 18S mRNA as a transfer efficiency and load control. The three stages of the menstrual cycle examined were (left to right), early luteal (EL), mid-luteal (ML) and late luteal (LL). The graph at the bottom summarizes the changes with black bars representing the relative change in BIGH3 signals.

culture procedures. Nonetheless, overall embryo implantation success rates remain low (<33%) in many areas, and a significant proportion of women receiving embryo transfers either require multiple procedures or never achieve a successful pregnancy (Report ASRM/SARTR, 2000). Various pathologies are associated with female infertility including endometriosis (Lu and Ory, 1995) and tubal disease (Hurst *et al.*, 2001). Studies have suggested that in some women with endometriosis or hydrosalpinges, uterine receptivity may be diminished (Lessey *et al.*, 1994; Meyer *et al.*, 1997). An underlying problem is the fundamental lack of useful predictors of the uterine state of receptivity for embryo implantation. It is generally agreed that the initial phase of human embryo implantation occurs during the mid-luteal phase with low success occurring during the early luteal phase (Lessey *et al.*, 1999). Similar observations have been made under well-controlled conditions in laboratory animals (Noyes *et al.*, 1963). Information already exists implicating various genes in the implanta-

Figure 4

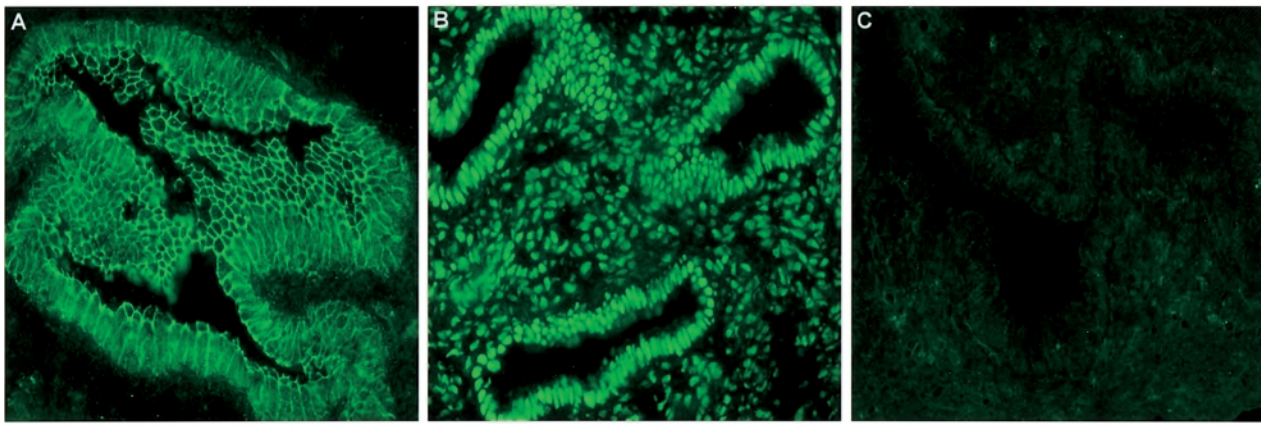

Figure 5

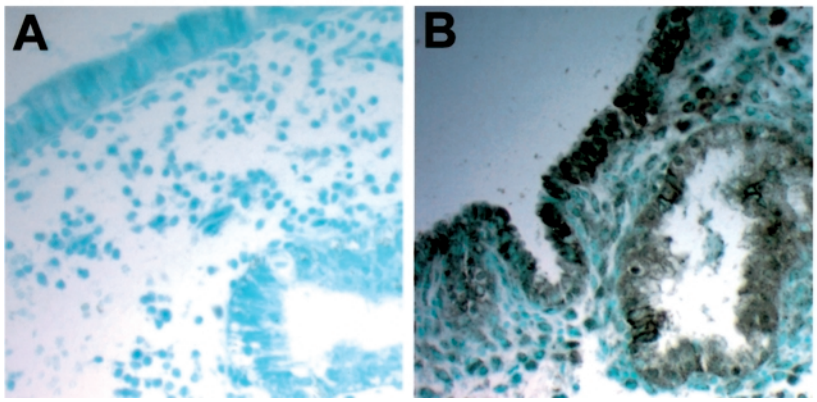

Figure 4. Immunofluorescence of mid-luteal human endometrial sections. (**A**) EP_1 receptor is abundantly expressed on the cell periphery of glandular epithelia and is absent from the surrounding stroma. (**B**) Leukotriene B_4 receptor is expressed intracellularly in both glandular epithelia and stroma. (**C**) Non-immune rabbit IgG does not cross-react with the tissue sections. Staining was performed as described in Materials and methods. Original magnification = 400×.

Figure 5. Immunohistochemical detection of claudin-4/CPE-R in mid-luteal human uterine epithelia. Immunostaining was performed as described in Materials and methods. (**A**) Staining with normal rabbit serum; (**B**) Staining with anti-claudin-4/CPE-receptor reveals strong specific staining of uterine epithelial but not stromal cells. Original magnification = 400×.

tion process (Salamonsen *et al.*, 2001). Furthermore, recent studies using microarray-based approaches have revealed changes in gene expression during human stromal cell differentiation (decidualization) *in vitro* (Popovici *et al.*, 2000). While useful information can be gained by comparing other uterine stages, e.g. proliferative or late luteal/menstrual phases, large changes in the hormonal milieu and gross, histologically evident changes in endometrial architecture indicate that many changes in gene expression occur and have little bearing on implantation-related processes (Parr and Parr, 1989). Thus, we chose to examine changes in gene expression that occur during the early to mid-luteal transition as a means of focusing on a set of changes in gene expression more closely tied to generation of a receptive uterine state. As a result, we report consistent changes in <6% of the genes surveyed. Those genes surveyed represent ~25–33% of the human transcriptome (Clavrie, 2001).

Confirmation of the observed changes relied not only upon repeated observations using the microarray approach, but also on results of previous studies identifying similar changes in expression of particular genes or gene products in the human endometrium. These included FGF pathway components (Sangha *et al.*, 1997), PP5 (Butzow *et al.*, 1986), decay accelerating factor/CD55 (Kaul *et al.*, 1996), MMP-7/matrilysin (Rodgers *et al.*, 1993) and cyclin B (Shiozawa *et al.*, 1996). Many other gene products in which changes were observed have been detected in human endometrium, although cycle-related changes have not been reported.

Complex changes in Wnt pathway regulators were observed during the early to mid-luteal transition. A large increase (10-fold) in Dickkopk1/DKK1, a Wnt pathway inhibitor (Semenov *et al.*, 2001), and a smaller (50%) decrease in LRP6, a Wnt co-receptor with frizzled (FrpHE) (Semenov *et al.*, 2001), occurred. This suggests that decreased Wnt-dependent signalling may occur. Paradoxically, these changes were paralleled by a large increase (>45-fold) in Wnt10B expression and large decrease (90%) in the soluble form of FrpHE, another Wnt pathway inhibitor (Salic *et al.*, 1997). Wnt-10b is reported to promote glandular development and hyperplasia in mammary tissue (Lane and Leder, 1997). Therefore, Wnt-10b also may promote endometrial glandular development that occurs during the luteal phase (Parr and Parr, 1989). FrpHE was shown to decrease during the luteal phase in an earlier report (Abu-Jawdeh *et al.*, 1999). In mice, estrogens suppress production of secreted frizzled related protein-2 (Das *et al.*, 2000). Therefore, nidatory estrogen might also be expected to suppress expression during the receptive phase in this species, although this has not been specifically determined. Whole endometrium, rather than isolated cell types, was used in these studies. The complexity

of the changes in Wnt pathway-related components suggests that compartmentalization of these changes may occur within the endometrium to balance responses within particular cell types. More detailed studies localizing the cellular source of these changes will be required to determine if this is the case.

A possible discrepancy with previous studies was the observed decrease in stromelysin-3/MMP-11. A careful study (Rodgers *et al.*, 1994) demonstrated stromelysin-3/MMP-11 transcript expression in proliferative and late luteal/early menstrual endometrium. The results of the present studies indicate that stromelysin-3/MMP-11 mRNA is increased during the early luteal phase, perhaps to support uterine extracellular matrix remodelling that occurs during the luteal phase (Parr and Parr, 1989; Lessey, 1998). Finally, many genes and ESTs displayed consistent changes that have not yet been studied in human endometrium and represent other avenues for more focused research.

Some genes expected to change in expression during the early to mid-luteal transition were not found to change in the current screen. In some cases, e.g. HOXA10 (Taylor *et al.*, 1998), these genes were not represented on the array. However, in other cases, e.g. progesterone receptor (Lessey *et al.*, 1996), the genes were represented. Gene products expressed by more than one cell type may undergo cell type-specific changes that are not of sufficient amplitude to be detected in the entire tissue. This is a potential shortcoming of the microarray approach, but could be overcome by the use of ancillary techniques such as laser capture or cell isolation prior to mRNA isolation. Alternatively, cell lines that mimic normal tissue components may also be useful to study specific cell types or to investigate regulation of select genes.

We chose several genes that consistently displayed large (>4-fold) changes in expression during the early to mid-luteal transition for further study. Northern blot analyses confirmed the large decrease in BIGH3 mRNA as well as increases in OPN and claudin-4/CPE receptor mRNA expression. Detailed examination of OPN mRNA and protein expression and distribution throughout the cycle has been examined recently and further confirms these observations (Apparao *et al.*, 2001). The increase in OPN expression complements increased expression of the OPN receptor complex, integrin $\alpha_V\beta_3$ in lumenal epithelium during the mid-luteal phase in humans (Lessey, 1998). Thus, an OPN:$\alpha_V\beta_3$ complex may occur at the apical surface of human uterine lumenal epithelium to promote embryo attachment, as suggested in other species (Johnson *et al.*, 1999). Claudin-4/CPE receptor is an epithelial protein and, presumably, is associated with uterine lumenal and/or glandular epithelium. Immunostaining revealed strong epithelial staining for claudin-4/CPE receptor at the mid-luteal phase confirming both the microarray and Northern blotting results and identifying the cellular site of protein accumulation. Clear demonstration of the function of this protein has not been performed; however, increases in the expression of this component of cell:cell junctional complexes appears to be part of a general increase in expression of other intercellular adhesion components, e.g. cadherins and associated proteins (Fujimoto *et al.*, 1996), that occurs during the transition from the prereceptive early luteal to the receptive mid-luteal phase. The prostaglandin EP_1 receptor also accumulated in epithelia during the mid-luteal phase indicating the site of accumulation of this gene product also identified by the microarray analysis. In contrast to the epithelial specific expression of these markers as well as of OPN (Apparao *et al.*, 2001), leukotriene B_4 receptor was expressed by both epithelial and stromal cells. Thus, while some markers display cell type-specific expression, others are more broadly expressed.

These studies provide the first transcriptome-based comparison of endometrial gene expression during the transition to the receptive uterine state in humans. From these results, it should be possible to construct much more limited gene arrays that can be used to determine the feasibility of using changes in gene expression for prediction of uterine receptivity as well as for the identification of underlying fertility defects.

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