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# Microarray Analysis of Uterine Epithelial Gene Expression during the Implantation Window in the Mouse

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**In mice, the uterus becomes transiently receptive to the hatched blastocyst on the day of implantation to allow its attachment to the luminal epithelium and subsequent invasion into the uterus. This uterine preparation for implantation is regulated by estradiol-17$\beta$ and progesterone, acting through their transcription factor receptors. Using ovariectomized mice treated with physiological regimens of these hormones, combined with methods to isolate RNA specifically from the uterine epithelium followed by transcriptome analysis on cDNA microarrays, 222 genes whose transcript abundance was specifically increased by estradiol-17$\beta$ and progesterone treatment were identified. Gene ontology analysis revealed an emphasis on genes involved with immune responses, extracellular matrix metabolism, and cell-to-cell communication. *In situ* hybridization to uterine sections isolated through the first 6 d of pregnancy identified novel sets of genes such as *Bach*, *Myd88*, *Cd14*, *Isg20*, and *Lrp2* whose expression was restricted to the uterine epithelium during the implantation window. Particularly notable was the expression of the mRNA for members of the signaling pathway from the Toll-like receptors to its downstream targets such as *Irg-1*. The identification of these genes showing a cell type hormonally regulated pattern of expression in the uterus suggests novel functions for them during implantation. (*Endocrinology* 147: 4904–4916, 2006)**

THE COORDINATED ACTIONS of female sex steroid hormones, progesterone ($P_4$) and estradiol-17$\beta$ ($E_2$) regulate uterine cell proliferation and differentiation in a spatiotemporal manner to establish the implantation window (1). In mice, $E_2$ synthesized at proestrus causes a synchronized wave of cell division in the luminal and glandular epithelium that is required for efficient embryo implantation. This $E_2$ also induces the LH surge required for ovulation and mating behavior, thus ensuring that the oocytes are fertilized to allow developmental synchrony with the uterine preparation for their implantation. Copulation results in the stimulation of a neuroendocrine loop that causes the secretion of prolactin by the pituitary and the maintenance of the corporea lutea. These synthesize the $P_4$ required for pregnancy that begins on the second to third day postcoitum. This $P_4$ blocks residual epithelial cell proliferation and induces the luminal epithelium to differentiate to allow embryo attachment and invasion. In addition, the $P_4$ induces a modest increase in stromal cell proliferation and priming the stromal cells to respond to the $E_2$ synthesized with a single wave of cell proliferation that occurs immediately before implantation. In fact, the uterus is hostile to implantation even in the progestinized state unless this so-called nidatory estrogen is synthesized, and without it the hatched blastocyst will enter a state of delay and not implant (2). This delay can be broken by a single injection of $E_2$ given to ovariectomized mice that have been maintained solely on $P_4$ (3). $E_2$ thus gives a transient permission for the embryo to attach and invade into the uterus. In normal mice, this so-called implantation window initiates on the morning of d 4 of pregnancy and lasts for approximately 18–24 h when the uterus becomes refractory to embryo implantation again by d 5 of pregnancy (3–5). Embryo implantation requires a series of well-coordinated events: close apposition of the blastocyst with the uterine luminal epithelium, adhesion of the trophectoderm to the luminal epithelium, invasion of the luminal epithelium, and subsequently the stroma by the trophectoderm. At the same time, the underlying stromal cells surrounding the implanting blastocysts undergo decidualization to form a protective and nutritive chamber for the blastocysts (5).

The effects in the uterus of $P_4$ and $E_2$ are primarily mediated through the progesterone receptor and estrogen receptor, respectively, which are ligand-inducible transcriptional factors (6, 7). Consequently it has been thought that these hormones regulate the expression of specific gene networks in distinct cell types and the products of these genes in turn mediate the hormone effects. Therefore, for many years there has been a search for genes that are regulated by these hormones. Many sex steroid hormone-regulated genes have been identified over the years by a mix of techniques including subtractive hybridization, candidate gene ap-

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Abbreviations: Bmp, Bone morphogenic; Lrp-2, low-density lipoprotein receptor-related protein; CSF, colony-stimulating factor; $C_T$, threshold cycle; DIG, digoxigenin; $E_2$, estradiol-17$\beta$; ECM, extracellular matrix; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GO, gene ontology; *Ihh*, Indian hedgehog; *Irg1*, immune response gene 1; LOWESS, locally weighted scatterplot smoothing; LPS, lipopolysaccharide; *Myd88*, myeloid differentiation primary response gene 88; NF-$\kappa$B, nuclear factor-$\kappa$B; $P_4$, progesterone; QRT-PCR, quantitative real-time PCR; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TLR, Toll-like receptor.

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proaches, or serendipity (5, 8–16). However, with the advent of large-scale expression profiling on DNA microarrays, the screening of large numbers of genes simultaneously has become possible, and several groups have performed such experiments to identify new classes of candidate genes whose functions might be crucial for implantation. These studies compared the differential uterine gene expression patterns from ovariectomized mice after different hormone treatments, hormone receptor inhibitor treatments, different stages of early pregnancy, or different uterine epithelial compartments or from human samples isolated at different stages of the menstrual cycle (17–21). However, despite considerable success in isolating novel patterns of gene expression. it should be recognized that the uterus is a complex tissue comprising many different resident cell types and also contains many cells of hematopoietic origin whose uterine abundance changes dramatically during early pregnancy (22). This heterogeneity adds complexity to the many of these analyses of gene expression patterns during the cyclical changes in the uterus.

Blastocysts make their first physical and physiological attachment with the apical surface of uterine luminal epithelium. However, the luminal epithelial cells represent only 5–10% of total uterine cells (23). Thus, it is probable that changes of gene expression in the luminal epithelium would be diluted out by analysis of the whole tissue homogenates, and many genes specifically regulated in this cell type would not be detected in these whole uterine global gene expression screens. In this study, therefore, we purified the uterine epithelium before isolating RNA and subjecting it to expression analysis using cDNA microarrays. Our microarray analysis revealed novel markers of uterine receptivity whose transcripts abundance is transiently increased during the implantation window. These provide valuable insights into the molecular mechanism underlying this complex physiological process by identifying the existence of several previously undocumented signaling pathways.

## Materials and Methods

### *Animal and treatments*

Adult female CD1 mice (Charles River Laboratories, Wilmington, MA) were maintained in the Association for Assessment and Accreditation of Laboratory Animal Care International Committee-approved animal barrier facility at the Albert Einstein College of Medicine (AECOM). All animal experiments were performed under National Institutes of Health guidelines for the care and use of laboratory animals and were approved by the AECOM animal use committee.

Hormones replacement experiments were performed as described before (24). Briefly, mice were ovariectomized at 8–10 wk of age under tribromoethanol anesthesia to remove the endogenous ovarian hormones. Two or three weeks later, they are primed with 100 ng $E_2$ for 2 d. After resting for another 2 d, groups of two to three mice were subjected to one of the following treatments: 1) a single injection of 50 ng $E_2$ given 4 d later to mimic the preovulatory estrogen during the estrus cycle ($E_2$ treatment); and 2) four daily injection of 1 mg $P_4$ with one injection of 50 ng $E_2$ with the last injection of $P_4$ on the fourth day to initiate the implantation window ($P_4E_2$ treatment).

### *Preparation of total RNA from uterine epithelium*

Three hours after the last hormone treatment, mice were killed, uteri removed and split longitudinally, and six uterine horns were vortexed with five Teflon beads (Small Parts, Inc., Miami, FL) in 1 ml extraction buffer [100 mM Tris-HCl (pH 7.4), 0.1 M NaCl, and 10 mM ribonucleoside vanadyl complex (New England Biolabs, Ipswich, MA)] for 2.5 min as described (25). Homogenates were filtered through nylon mesh, and the residual tissues and beads were washed once with 1 ml extraction buffer and filtered again through nylon mesh to obtain a pure preparation (≥95%) of uterine epithelial cells. Total RNA was isolated from the uterine epithelium using the guanidinium isothiocyanate method (26) as modified (27). Briefly, the 2-ml homogenate was mixed immediately with 4 ml of 6 M guanidinium isothiocyanate, 37.5 mM sodium citrate (pH 7.0), 0.75% (vol/vol) *N*-lauroylsarcosine, and 0.15% (vol/vol) $\beta$-mercaptoethanol. The lysates were then layered onto a 4-ml 5 M CsCl cushion centrifuged at 100,000 × $g_{av}$ for 18 h at 18 C. Approximately 20–30 $\mu$g total RNA was isolated from each group of three mice, and the integrity of total RNA was monitored using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples were pooled to obtain sufficient RNA (100 $\mu$g/slide) for labeling and analysis on the cDNA microarrays and Northern blotting as described below.

### *Microarray analysis*

Mouse cDNA microarray slides were obtained from the microarray facility at the AECOM. Each array encompasses 27,396 mouse cDNA sequence-verified clones from Incyte Genomics (Wilmington, DE), the National Cancer Institute, and Integrated Molecular Analysis of Genomes (28). Microarray analysis was performed on three separate microarray slides following the standard procedure provided by the AECOM microarray facility. Briefly, for each hybridization reaction, the first-strand cDNA probes were generated by reverse transcription of 100 $\mu$g total RNA through the incorporation of cy3-deoxyuridine 5-triphosphate ($E_2$-treated sample) or cy5-deoxyuridine 5-triphosphate ($P_4E_2$-treated sample). The two cDNA probes were mixed, denatured at 94 C, and hybridized to an array slide overnight at 50 C. After rinsing in 1× saline sodium citrate (SSC)/0.1% (wt/vol) sodium dodecyl sulfate (SDS) for 20 min and twice in 0.2× SSC/0.1% (wt/vol) SDS for 20 min, the slides were dried and scanned using a custom-built laser scanner.

### *DNA microarray quality control, normalization, and data analysis*

The absolute intensities for both channels of each spot on the array were obtained using GenePix 3.0 software (Axon Instruments, Union City, CA). An MA plot was used to represent the (R, G) data, where R = red for cy5 ($P_4E_2$-treated sample), G = green for cy3 ($E_2$-treated sample), and log intensity ratio $M = \log_2 (R/G) = \log_2 R - \log_2 G$ was plotted against mean log intensities $A = \log_2 \sqrt{R \times G} = (\log_2 R + \log_2 G)/2$ for each array, as described by Yang *et al.* (29). Scale normalization was performed for each array using the locally weighted scatterplot smoothing (LOWESS) procedure of the microarray package in Bioconductor. The efficiency of LOWESS normalization was assessed by monitoring M-A plots for each array before and after LOWESS normalization. In our experiments analyzing three arrays, the spots with the average intensity A of 7 or more ($2^7 = 128$) and the fold change M of 1 or more ($R/G \geq 2$) or M of −0.83 or less ($G/R \geq 1.8$) on at least two arrays was chosen as the transcripts whose abundance was significantly up-regulated and down-regulated and kept for the further analysis and validation. Previous validation in our laboratory had shown, using repeated hybridization between the same samples labeled with either cy5 or cy3 (in either direction) as described above, that 1.8-fold differences represented 2 SD from the mean of variation in signal detection between these same two samples (Mackler, A., and J. W. Pollard, unpublished data). Thus, we have more than 95% confidence that a change of 1.8-fold represents a real change in gene expression.

### *Quantitative real-time PCR (QRT-PCR)*

To verify the expression data obtained from the microarray analysis, QRT-PCR analysis of selected genes was performed on DNA Engine Opticon2 (Bio-Rad, Hercules, CA) using SYBR Green qPCR supermix UDG (Invitrogen Corp., Carlsbad, CA). Supplemental Table 2, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org, lists the primer sequences and amplicon size. Briefly, three independently isolated aliquots of 5 $\mu$g total RNA derived from the uterine epithelium after different hormone treat-

ments were reverse transcribed into cDNA. This cDNA was used for real time PCR as follows: 95 C for 15 min followed by 40 cycles of 94 C for 15 sec, 60 C for 30 sec, and 72 C for 30 sec. Each time the PCR was performed in a 20-$\mu$l volume in three different wells. Every PCR experiment for each gene was repeated in triplicate. The changes in gene expression were calculated by median threshold cycle ($C_T$) and then normalized for the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts, using the $2^{-\Delta\Delta CT}$ methods (30). All $2^{-\Delta\Delta CT}$ values were logarithmically transformed to obtain normal distributed data. Real-time PCR data are presented as means ± SE.

### *In situ hybridization*

Digoxigenin (DIG)-labeled riboprobes were prepared using a DIG RNA labeling kit (Roche Diagnostics, Indianapolis, IN) following the manufacturer's protocol. Natural pregnancies were followed after detection of the vaginal plug, which was designated as d 1 of pregnancy and uteri isolated on the appropriate day. Uterine tissues were collected from mice of d 1–6 pregnancy and frozen in optimal cutting compound (Tissue-Tek) in liquid $N_2$. Twelve-micrometer cryosections were cut and fixed in 0.1 M sodium phosphate-buffered 4% (wt/vol) paraformaldehyde (pH 7.4), at 4 C for 10 min. They were rinsed twice in PBS, followed by acetylation in the solution containing 0.25% (vol/vol) acetic anhydride, 1.5% (vol/vol) triethanolamine, and 0.42% (vol/vol) HCl for 10 min. The slides were washed twice in PBS, prehybridized at 58 C for 2 h, and hybridized in hybridization buffer (50% formamide, 5×SSC, 5× Denhardt's, and 1 mg/ml sperm DNA) containing antisense or sense RNA probes at 58 C overnight. The slides were washed in 2× SSC at 58 C for 30 min; 2× SSC at 37 C for 10 min; and digested with RNase A in 2× SSC at 37 C for 30 min. Slides were subsequently washed sequentially with 2× SSC at 37 C for 10 min, 0.2× SSC at 58 C for 30 min twice, and B1 solution [0.1 M Tris (pH 7.6), 0.15 M NaCl] for 5 min. The slides were incubated with 1:5000 anti-DIG antibody in B2 solution (0.5% Boehringer blocking regent in B1) for 2 h followed by equilibration in B3 solution [0.1 M Tris (pH9.5), 0.1 M NaCl, 50 mM $MgCl_2$] for 5 min. Finally the slides were developed with 4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate (Roche Diagnostics) overnight in the dark before the reaction was stopped by Tris-EDTA. For visualization the slides were counterstained with 1% (wt/vol) methyl green.

### *Northern blot analysis*

Northern blots were performed as described with some modifications (31). Briefly, 15–20 $\mu$g of total uterine epithelial RNA were separated by formaldehyde-agarose gel electrophoresis and transferred to nylon membranes using the Turbo blotter method (Schleicher & Schuell, Keene, NH). The RNA was fixed by UV cross-linking before prehybridization in hybridization buffer [25 mM $KPO_4$ (pH 7.4), 5× SSC, 5× Denhardt's solution, 10 $\mu$g/ml salmon sperm DNA, 50% formamide, 1% SDS] for 45 C for 1–3 h. cDNA probes for the genes of interest were labeled with $^{32}$P dCTP using the Rediprime II random prime labeling system (GE Healthcare Life Sciences Biotech, Piscataway, NJ). The membranes were hybridized with these $^{32}$P-labeled cDNA at the concentration of $10^6$ cpm/ml at 45 C overnight. After washing twice in 1× SSC/0.1% SDS buffer and twice in 0.25× SSC/0.1% SDS for 15 min at 45 C, the membranes were exposed to x-ray film or a phosphor screen. The final results were normalized by the signal of GAPDH as total RNA loading control.

## Results

### *Gene expression profiling defines transcripts whose abundance is increased in the uterine epithelium after $P_4E_2$ treatment*

The hormonal regimens used in these studies parallel the physiological situation in which 3 d of $P_4$ injection permits the uterine luminal epithelia to differentiate into a prereceptive state, and the combination of $P_4$ and $E_2$ on the fourth day induces the receptive state. In contrast, a single injection of $E_2$ mimics the preovulatory $E_2$ surge during the estrus cycle. We chose an early time period after the hormone treatment because we also wanted to identify genes that are involved in the $E_2$-regulated and $P_4$-inhibited uterine epithelial cell proliferation, and earlier studies had shown that $P_4$ could inhibit the $E_2$ induction of cell division only if given within 3 h of $E_2$ (32). Furthermore, we reasoned that early changes in gene expression were likely to instruct the luminal epithelium to prepare for the blastocyst implantation that occurred 7–10 h after nidatory $E_2$.

To identify genes that are differentially expressed 3 h after $P_4E_2$ *vs.* $E_2$ treatment, uterine epithelia cell extracts with more than 95% purity and consisting mostly of luminal epithelium were obtained from ovariectomized mice that had been given the different hormonal treatments. Total RNA extracted from this preparation was subjected to transcriptome analysis using the AECOM mouse cDNA microarrays that contains approximately 27,000 identified sequences.

To generate reproducible gene expression data, three independent microarray experiments were performed. The differential gene expression pattern of uterine epithelia after he different hormone treatments was visualized by the MA plot, where log intensity ratios $M = \log_2(R/G) = \log_2 R - \log_2 G$ was plotted against mean log intensities $A = (\frac{1}{2} \log_2 \sqrt{RG}$ (Fig. 1A). After LOWESS normalization to remove systematic variation

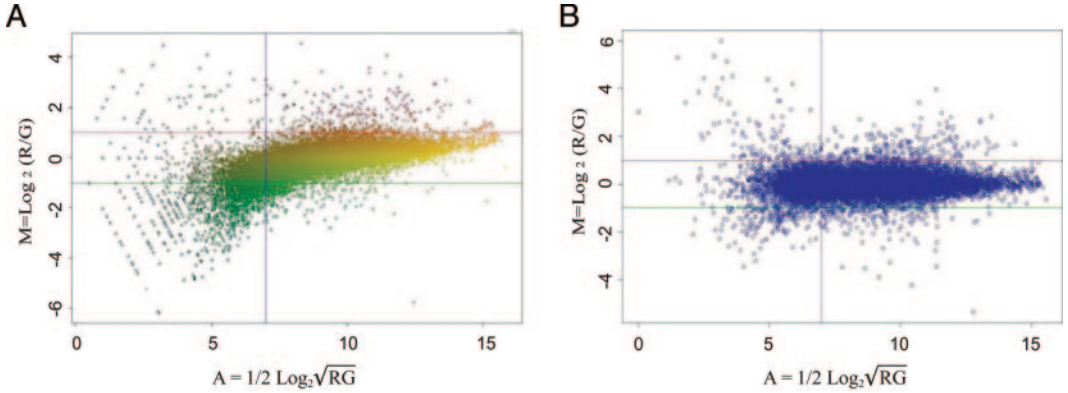


FIG. 1. MA scatter plots of the cDNA microarray data comparing $P_4E_2$- and $E_2$-treated groups. Microarray results of a single representative experiment are shown by MA scatter plot before (A) and after (B) LOWESS normalization. The x-axis shows the A value representing the mean log intensity, and the y-axis indicates the M value representing the log ratio of intensity. The *red line* shows M = 1 (R/G = 2), and the *green line* indicates M = −1 (R/G = 0.5). The *blue line* indicates the A = 7.

including dye biases, the total group of genes displayed a symmetrical distribution on the MA plot (Fig. 1B), indicating the efficiency of the LOWESS normalization. Only the expression of a small number of genes varied significantly between the two cohybridized uterine samples. We applied a threshold of A of 7 or greater ($2^7 = 128$) combined with M of 1 or greater ($R/G \geq 2$) or M of $-0.8$ or less ($G/R \leq 1.8$) to filter the differentially expressed genes. This analysis revealed 222 and 208 transcripts whose abundance is increased or decreased, respectively, in the $P_4E_2$-treated uterine samples, compared with $E_2$-treated ones. This current study focused on those whose abundance increases. In those whose abundance decreases, we identified a cohort of approximately 20 involved in the regulation of DNA synthesis, and these have been investigated at a biochemical level in another study (Pan, H., Y. Deng, and J. W. Pollard, submitted for publication). A complete list of those genes whose transcripts increase in abundance is presented in supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online Web site at http://endo.endojournals.org, although those analyzed in greater detail (Table 1) will be described below.

### *Categorization and validation of genes whose transcript abundance is significantly increased.*

Gene ontology (GO) (http://www.geneontology.org) annotation was used for categorization and deep analysis of our microarray data. A comprehensive database that integrated GO terms, definitions, and ontologies with AECOM microarray data set was created using Microsoft Access software. The hierarchical directed acyclic graph structure of GO was kept intact, and deep analysis was focused on the Biological Process subcategory. One hundred forty-nine known genes were classified into 11 subcategories of biological process after removal of the 73 unknown genes (32.8%) that included expressed sequence tags and Institute of Physical and Chemical Research (Rikagaku Kenkyusho) cDNA sequences (Table 1 and Fig. 2).

The four largest categories were extracellular matrix (ECM)/cell adhesion and tissue remodeling (29, 19.4%), signal transduction (23, 15.4%), enzymes related to amino acid and protein metabolism (17, 11.4%), and immune-related molecules (15, 10.0%). We also observed other groups including those encoding transport proteins, carbohydrate metabolism enzymes, transcription factors, and cytoskeleton proteins (Table 1). We further validated the microarray data by performing QRT-PCR for a selected subset of genes that show significant up-regulation on the arrays in three independently isolated uterine samples from each group. The expression of all six genes, *Myd88*, *Cd14, Isg20,* (immune function, Table 1), *Lrp2* (signaling transduction, Table 1), *Bach-pending* (metabolism, Table 1), and *Sultn* (ECM/cell adhesion and tissue remodeling, Table 1), shows a significant up-regulation in the $P_4E_2$-treated group, compared with the $E_2$-treated one, consistent with the results from the microarray experiment (Fig. 3). There were also several genes at the top of our list whose expression had previously been identified as implantation-related genes specifically expressed in the uterine luminal epithelium. These included immune response gene 1 (*Irg1*) (15, 21, 33), Indian hedgehog (*Ihh*) (34), monoamine oxidase B, histidine decarboxylase (11), Sultn (21), cathepsin D (35), and calbindin-28 K (36). These QRT-PCRs, together with the coincidence of up-regulation for six genes whose induction has already published (indicated as known in Table 1) and with the *in situ* hybridization and Northern data shown below, validate the microarray data. Thus, we can conclude with a high degree of confidence that $P_4E_2$ treatment increases the transcript abundance of at least 222 genes in the uterine epithelium.

### *Induction of transcripts of immune-related genes in the uterine epithelium coincident with the implantation window*

The transcripts of several of those genes, whose functions in other circumstances are associated with immune responses, were increased as assessed by our microarray analysis. Of particular note were several components involved in the Toll-like receptor (TLR) signaling pathway. In mammals, bacterial lipopolysaccharide (LPS) triggers an innate immune response by binding to the heteromeric receptor complex that consists of CD14, MD2, and TLR4 with the resultant recruitment of the cytoplasmic adaptor molecule, myeloid differentiation primary response gene 88 (*Myd88*). This signaling pathway ultimately activates the transcription factor nuclear factor-κB (NF-κB) and leads to the release of proinflammatory cytokines such as IL-6, TNFα, and IL-1α (37). The genes in this pathway whose transcript abundance is increased include *Myd88*, *Cd14*, and *Irg1*. Originally identified as a LPS-induced downstream gene in macrophages, *Irg1* has shown significant up-regulation in uterine luminal epithelium during the implantation window (14). To assess the physiological regulation of the other two genes and further validate the microarray data, the expression pattern and localization during the periimplantation period of naturally mated mice was investigated by *in situ* hybridization of longitudinal sections of uteri from d 2–6 of pregnancy (Fig. 4, A and B). Using antisense probes to *Myd88*, there was no detectable signal in uterine sections on d 2 and 3 of pregnancy (Fig. 4A). However, there was a dramatic induction of *Myd88* mRNA on d 4 that was present uniformly throughout the luminal epithelium but was absent from the glandular epithelium (Fig. 4A). After the blastocyst attachment reaction that induces the beginning of decidualization on d 5 and after implantation on d 6, the *in situ* signal for Myd88 mRNA disappeared. No hybridization signal for *Myd88* was observed in the sense control uterine sections (Fig. 4A).

Examination of the expression of the TLR-4 coreceptor *Cd14* mRNA by *in situ* hybridization also demonstrated a transient induction of the mRNA during the periimplantation period. However, when compared with Myd88 mRNA, this had a distinct although overlapping temporal and spatial pattern of expression (Fig. 4B). Cd14 mRNA was expressed very weakly in the luminal and glandular epithelium on d 2 (Fig. 4B). Its expression reached a peak on d 3 in both compartments before declining on d 4 (Fig. 4B) with expression being lost by d 5 and 6 of pregnancy (Fig. 4B). Hybridization using sense probes were consistently negative (Fig. 4B).

Another interesting gene whose transcript abundance increased in the immune-related gene list is interferon-stimulated gene, *Isg20*. ISG20, a 3′ 5′ exonuclease with specificity for single-

**TABLE 1.** Genes whose transcript abundance in the mouse uterine epithelium is up-regulated by $P_4E_2$, compared with $E_2$

| Gene | Description | ID | Fold-up | SD | Validation |
|---|---|---|---|---|---|
| Immune-related molecules | | | | | |
| *Irg1* | Immunoresponsive gene 1 | AI323667 | 9.692 | 6.517 | Known |
| *Myd88* | Myeloid differentiation primary response gene 88 | AU041653 | 3.767 | 0.078 | Validated |
| *Alox12e* | Arachidonate lipoxygenase, epidermal | C88083 | 3.667 | 1.020 | |
| *Isg20* | Interferon-stimulated protein | AU041869 | 3.425 | 0.563 | Validated |
| *Cln5* | Ceroid-lipofuscinosis, neuronal 5 | AW542388 | 3.327 | 0.459 | |
| *Arg2* | Arginase type II | AU043044 | 3.198 | 0.309 | |
| *Emb* | Embigin | AW536222 | 2.932 | 0.531 | |
| *Cd14* | CD14 antigen | AA396117 | 2.517 | 0.469 | Validated |
| *Daf1* | Decay accelerating factor 1 | AU016251 | 2.175 | 0.173 | |
| *Islr* | Immunoglobulin superfamily containing leucine-rich repeat | AA537116 | 2.100 | 0.347 | |
| *Ltb4dh* | Leukotriene B4 12-hydroxydehydrogenase | C78257 | 2.072 | 0.294 | |
| *Asl* | Argininosuccinate lyase | AU019411 | 2.667 | 0.694 | |
| ECM/cell adhesion and tissue remodeling | | | | | |
| *Sultn* | *N*-sulfotransferase | AA275042 | 8.541 | 4.775 | Validated |
| *Serpina1e* | Serine (or cysteine) proteinase inhibitor, clade A, member 1e | AU021496 | 6.343 | 1.391 | |
| *Col3a1* | Procollagen, type III, $\alpha$ 1 | AW552978 | 5.106 | 1.577 | |
| *Comp* | Cartilage oligomeric matrix protein | AA064293 | 3.797 | 0.257 | |
| *Cnn3* | Calponin 3, acidic | AI465251 | 3.432 | 0.794 | |
| *Jam2* | Junction adhesion molecule 2 | AU016127 | 3.757 | 0.970 | |
| *Cldn3* | Claudin 3 | AU040223 | 3.194 | 0.823 | |
| *Col5a2* | Procollagen, type V, $\alpha$2 | AA034564 | 3.005 | 0.991 | |
| *Nid1* | Nidogen 1 | AW536426 | 2.764 | 0.523 | |
| *Spint1* | Serine protease inhibitor, Kunitz type 1 | C77627 | 2.943 | 0.104 | |
| *Cd34* | CD34 antigen | AW546851 | 2.469 | 0.392 | |
| *Vim* | Vimentin | AW548722 | 2.332 | 0.193 | |
| *Sparc* | Secreted acidic cysteine rich glycoprotein | W34157 | 2.355 | 0.304 | |
| *Tm4sf9* | Transmembrane 4 superfamily member 9 | AU024142 | 2.165 | 0.270 | |
| *Mmp7* | Matrix metalloproteinase 7 | AU017967 | 2.243 | 0.435 | |
| *Sultx1-pending* | Sulfotransferase related gene $\times$ 1 | W16116 | 2.340 | 1.179 | |
| *Add3* | Adducin 3 ($\gamma$) | AA178305 | 2.163 | 0.398 | |
| *Mmp2* | Matrix metalloproteinase 2 | AI464548 | 2.098 | 0.041 | |
| *Lamc3* | Laminin $\gamma$3 | AI414335 | 2.079 | 0.173 | |
| *Adam12* | A disintegrin and metalloproteinase domain 12 (meltrin-$\alpha$) | W65081 | 2.028 | 0.019 | |
| Signaling transduction | | | | | |
| *Lrp2* | Low-density lipoprotein receptor-related protein 2 | AU043137 | 5.416 | 0.423 | Validated |
| *Sbk-pending* | SH3-binding kinase | W67049 | 3.584 | 0.716 | |
| *Ptpn11* | Protein tyrosine phosphatase, nonreceptor type 11 | AI323459 | 3.513 | 1.044 | |
| *F2r* | Coagulation factor II (thrombin) receptor | AI464520 | 2.713 | 0.142 | |
| *Lrpap1* | Low-density lipoprotein receptor-related protein-associated protein 1 | AW557574 | 2.587 | 0.355 | |
| *Apc* | Adenomatosis polyposis coli | AU020952 | 1.989 | 0.858 | |
| *Arhb* | Ras homolog gene family, member AB | W33989 | 2.371 | 0.057 | |
| *Ihh* | Indian hedgehog | AA245525 | 2.314 | 0.262 | Known |
| *Ptger4* | Prostaglandin E receptor 4 (subtype EP4) | AU021621 | 2.303 | 0.181 | |
| *Marcks* | Myristoylated alanine rich protein kinase C substrate | AA170009 | 2.213 | 0.205 | |
| *Tsc1* | Tuberous sclerosis 1 | AA544963 | 2.174 | 0.010 | |
| *Igf1* | IGF-I | AU041343 | 2.201 | 0.125 | |
| *Itpr5* | Inositol 1,4,5-triphosphate receptor 5 | C86832 | 2.050 | 0.105 | |
| *Fgfr2* | Fibroblast growth factor receptor 2 | AW556123 | 2.206 | 0.571 | |
| *Igfbp3* | IGF binding protein 3 | AW557928 | 2.034 | 0.083 | |
| *Tgfbr3* | TGF, $\beta$-receptor III | AI426128 | 2.031 | 0.174 | |
| *Fstl* | Follistatin-like | AA242611 | 2.060 | 0.386 | |
| *Gnai1* | Guanine nucleotide binding protein, $\alpha$-inhibiting 1 | AU046200 | 2.136 | 0.122 | |
| *Entpd2* | Ectonucleoside triphosphate diphosphohydrolase 2 | AU018470 | 2.223 | 1.110 | |
| Enzyme/amino acid and protein metabolism | | | | | |
| *Maob* | Monoamine oxidase B | AA241899 | 5.190 | 1.238 | Known |
| *Hdc* | Histidine decarboxylase | AA118747 | 5.219 | 2.397 | Known |
| *Bach-pending* | Brain acyl-CoA hydrolase | AW550836 | 4.291 | 0.554 | Validated |
| *Gsto1* | Glutathione S-transferase omega 1 | AU040215 | 3.510 | 0.507 | |
| *Gstm1* | Glutathione S-transferase, $\mu$1 | AW553206 | 3.229 | 0.219 | |
| *Cbs* | Cystathionine $\beta$-synthase | AA277347 | 3.123 | 0.663 | |
| *Ogt* | *O*-linked *N*-acetylglucosamine (GlcNAc) transferase (UDP-*N*-acetylglucosamine:polypeptide-*N*-acetylglucosaminyl transferase) | AW548125 | 2.556 | 0.267 | |
| *Gstm4* | Glutathione S-transferase, $\mu$4 | AW555156 | 2.462 | 0.233 | |
| *Gstm6* | Glutathione S-transferase, $\mu$6 | AW555644 | 2.335 | 0.556 | |
| *Ccbl1* | Cysteine conjugate-$\beta$ lyase | AU020349 | 2.167 | 0.082 | |
| *Glul* | Glutamate-ammonia ligase (glutamine synthase) | AA011759 | 2.122 | 0.200 | |
| *Entpd2* | Ectonucleoside triphosphate diphosphohydrolase 2 | AU018470 | 2.223 | 1.110 | |
| *Gldc* | Glycine decarboxylase | AW537411 | 2.044 | 0.211 | |
| *Srd5a1* | Steroid 5$\alpha$-reductase 1 | AI414607 | 3.389 | 0.861 | |
| *Rdh11* | Retinol dehydrogenase 11 | AU016487 | 2.007 | 0.075 | |
| Transport protein | | | | | |
| *Fxyd4* | FXYD domain-containing ion transport regulator 4 | AU046167 | 3.953 | 1.606 | |

**TABLE 1.** *Continued*

| Gene | Description | ID | Fold-up | SD | Validation |
|---|---|---|---|---|---|
| *Slc2a12* | Solute carrier family 2, member 12 | AA051207 | 3.698 | 0.904 | |
| *Slc5a10* | Solute carrier family 5 (sodium/glucose cotransporter), member 10 | C80370 | 3.497 | 0.585 | |
| *Tmc5* | Transmembrane channel-like gene family 5 | AU040460 | 2.653 | 0.382 | |
| *Abca1* | ATP-binding cassette, subfamily A (ABC1), member 1 | AA063753 | 2.575 | 0.302 | |
| *Slc31a2* | Solute carrier family 31, member 2 | AW555365 | 2.262 | 0.270 | |
| *Aqp1* | Aquaporin 1 | AA241281 | 2.176 | 0.187 | |
| *Crot* | Carnitine *O*-octanoyltransferase | AA239277 | 2.047 | 0.320 | |
| Carbohydrate metabolism | | | | | |
| *Ggta1* | Glycoprotein galactosyltransferase $\alpha$1, 3 | AA175441 | 4.241 | 1.211 | |
| *Pfkfb3* | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 | AU021476 | 3.397 | 0.640 | |
| *H6pd* | Hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase) | AA537509 | 2.661 | 0.333 | |
| Transcriptional factors | | | | | |
| *Pnrc2* | Proline-rich nuclear receptor coactivator 2 | AA444485 | 3.190 | 2.247 | |
| *Npas2* | Neuronal PAS domain protein 2 | AU021431 | 2.986 | 1.821 | |
| *Bteb1* | Basic transcription element binding protein 1 | AA036347 | 2.137 | 0.191 | |
| *Klf15* | Kruppel-like factor 15 | AA060858 | 2.028 | 0.223 | |
| *Ebf1* | Early B-cell factor 1 | AA008591 | 2.165 | 0.935 | |
| *Ly108* | Lymphocyte antigen 108 | AA472962 | 2.250 | 0.180 | |
| *Crtr1-pending* | Tcfcp2-related transcriptional repressor 1 | AU043264 | 2.505 | 0.339 | |
| *Cited4* | Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 4 | AU019445 | 2.412 | 0.413 | |
| Cytoskeleton protein | | | | | |
| *Nde1* | Nuclear distribution gene E homolog 1 (A nidulans) | AU042936 | 3.207 | 0.242 | |
| *Tmod2* | Tropomodulin 2 | C86219 | 2.798 | 0.260 | |
| *Mtap2* | Microtubule-associated protein 2 | AA386889 | 2.817 | 0.535 | |
| *Cnn3* | Calponin 3, acidic | C86934 | 3.432 | 0.794 | |
| *Add3* | Adducin 3 ($\gamma$) | AA178305 | 2.163 | 0.398 | |
| *Mfap5-pending* | Microfibrillar associated protein 5 | AA037995 | 2.054 | 0.159 | |
| Cell proliferation, differentiation, and apoptosis | | | | | |
| *Ctsd* | Cathepsin D | AW554219 | 5.384 | 1.698 | Known |
| *Tde1* | Tumor differentially expressed 1 | AW553363 | 2.499 | 0.382 | |
| *Ccng1* | Cyclin G1 | AU019834 | 2.218 | 0.276 | |
| *Gilz* | Glucocorticoid-induced leucine zipper | W66757 | 2.037 | 0.283 | |
| *Btg2* | B cell translocation gene 2, antiproliferative | AA154848 | 2.050 | 0.250 | |
| *Smtn* | Smoothelin | AA498288 | 2.176 | 0.147 | |
| Metal-binding protein | | | | | |
| *Calb1* | Calbindin-28K | AU041945 | 4.333 | 1.266 | Known |
| *Mt2* | Metallothionein 2 | AW539788 | 3.645 | 0.354 | |
| *Mt1* | Metallothionein 1 | AW544811 | 3.387 | 0.329 | |
| *Calb3* | Calbindin 3 (vitamin D-dependent calcium binding protein) | AU040803 | 3.185 | 0.421 | |
| Function unknown | | | | | |
| *D7Rp2e* | DNA segment, Chr 7, Roswell Park 2 complex, expressed | AU016790 | 5.494 | 0.677 | |
| *Upa* | Uterine-specific proline-rich acidic protein | W17866 | 4.999 | 2.340 | |
| *Nme7* | Nonmetastatic cells 7, protein expressed in | AW544502 | 4.149 | 0.426 | |
| *Ovgp1* | Oviductal glycoprotein 1 | AU041578 | 3.059 | 0.933 | |
| *Mic2l1* | MIC2 (monoclonal Imperial Cancer Research Fund 2)-like 1 | AW548191 | 3.272 | 0.549 | |
| *Pop4-pending* | POP4 (processing of precursor, *S. cerevisiae*) homolog | AU042817 | 3.141 | 0.465 | |
| *cobl* | Cordon-bleu | AI413789 | 3.095 | 0.399 | |
| *Map17-pending* | Membrane-associated protein 17 | AU043587 | 2.873 | 0.970 | |
| *Pfpl* | Pore forming protein-like | AW544632 | 2.721 | 1.055 | |
| *Kap* | Kidney androgen regulated protein | AW551536 | 2.488 | 0.093 | |
| *D8Bwg1320e* | DNA segment, Chr 8, Brigham & Women's Genetics 1320 expressed | AA139627 | 2.213 | 0.085 | |
| *Olfm1* | Olfactomedin 1 | AW548326 | 2.177 | 0.663 | |
| *Tor3a* | Torsin family 3, member A | AI414415 | 2.076 | 0.415 | |
| *Pepp2-pending* | Phosphoinositol 3-phosphate-binding protein-2 | AA064296 | 2.037 | 0.535 | |
| *Tbrg4* | TGF$\beta$-regulated gene 4 | W87077 | 2.241 | 0.087 | |

Transcript abundance elevated by $P_4E_2$, compared with $E_2$ in the mouse uterine epithelium.

stranded RNA, was recently identified as an interferon-induced protein that represents a novel pathway that interferes with viral infection and propagation (38). Examination of *Isg20* mRNA expression during the periimplantation by *in situ* hybridization demonstrated that the induction of *Isg20* mRNA in the uterine epithelium was concomitant with the initiation of the implantation window. Essentially no signal was present in the luminal and glandular epithelium on d 2, but at d 3, it began to increase with a dramatic induction on d 4 (Fig. 4C). Expression was initially detected in both luminal and glandular epithelium on d 3, but on d 4, coincident with the peak expression, there was a relative loss in the glandular epithelium (Fig. 4C). On d 5 and 6, the *Isg20 in situ* signal decreased rapidly and disappeared completely from the luminal epithelium and could not be detected in the decidual zone (Fig. 4C).

### *Genes associated with adhesion are regulated at implantation*

Nidatory estrogen causes the uterine epithelial cells to undergo significant morphological changes including apical microvilli retraction and the generation of pinopodia accompanied by the loss of their polarized characteristics (3). Consistent with these observations, the largest group of genes whose transcripts were increased in abundance by $P_4E_2$ in-

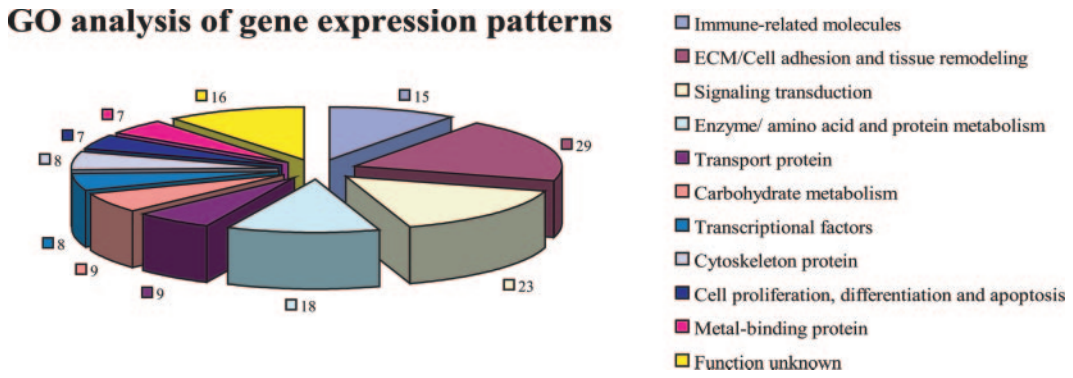


FIG. 2. GO annotation (category) of genes up-regulated by $P_4E_2$. The *different colored segments* of the pie chart represent the relative proportion of the up-regulated transcripts organized into 11 functional categories by GO notation.

cluded those that encode components involved in cell-to-cell communication, tight and adherens junctional contacts, and components of the ECM. Examples in the former group include transcripts for junction adhesion molecule 2, claudin 3, and calponin 3, whereas in the latter group, examples included the fibrous protein procollagen types III and V, laminin, nidogen, and cartilage oligomeric matrix protein. In addition, at the top of this category, transcripts encoding the enzyme, *N*-sulfotransferase, showed the highest induction by $P_4E_2$ as was confirmed by RT-PCR described above (Table 1 and Fig. 3). This enzyme catalyzes the deacetylation and sulfation of *N*-acetyl-D-glucosamine residues of heparin sulfate, a key step in the biosynthesis of heparin sulfate proteoglycan (39). Our previous studies had identified this gene as being preferentially expressed in the uterine epithelium (21).

### *Signaling pathways, transcription factors, and lipid metabolism enzymes induced in the uterine epithelia at implantation*

Successful implantation requires precise cell-cell communication between the hatched blastocyst and receptive uterus. The transcript abundance of several genes involved in growth factor signaling pathways was up-regulated by $P_4E_2$, *i.e.* TGF$\beta$ receptor 3, fibroblast growth factor receptor 2, and IGF-I and IGF-binding protein 3 (Table 1). Furthermore, several signaling pathways essential for pattern formation during embryogenesis were expressed in the uterine epithelium during the implantation window. These included *Apc*, an important component of Wnt/$\beta$catenin signaling pathway, and *Ihh*, whose product is the mitogen of the Indian hedgehog-signaling pathway (Table 1). These data are consistent with previous observations (40, 41). In addition, the mRNA for low-density lipoprotein receptor-related protein, *Lrp-2* (42), was also up-regulated by $P_4E_2$ in the uterine epithelium. Analysis by *in situ* hybridization of the uterus from d 2 to 6 of pregnancy showed no expression of *Lrp-2* on d 2 followed by an induction on d 3 in both the luminal and glandular epithelia (Fig. 4D). On d 4, it was still strongly expressed in the luminal epithelium, but expression was lost from the glandular epithelia (Fig. 4D). On d 5 and 6, *Lrp-2* expression shifted from the luminal epithelia to the decidualizing stroma surrounding the implanting embryos. There was no significant signal detected at any stage using the sense control probe (Fig. 4D).

Interestingly, there were also a group of transcription factors whose transcript abundance was increased by the $P_4E_2$ treatment. These included seven transcription factors, *Cited4, Pnrc2, Bteb1, Npas2, Klf15, Ebf1*, and *Crtr1-pending*. These factors may be amplifiers of the original transcriptional response acting downstream from the steroid hormone receptors.

The transcript abundance of several genes involved in metabolism, particularly lipid metabolism, was also in-

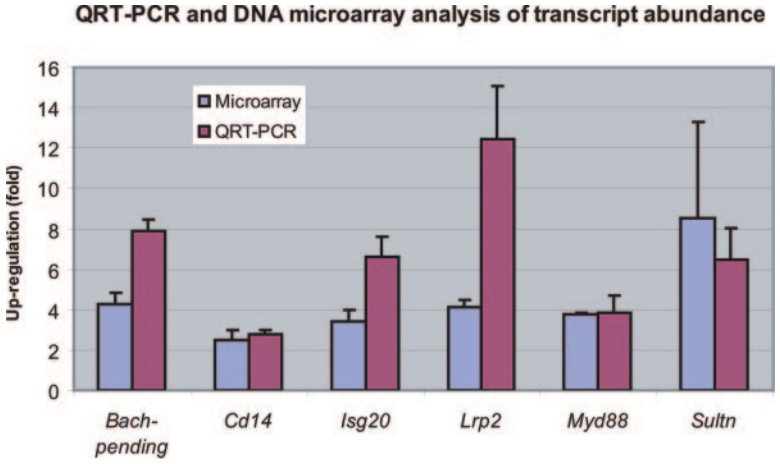


FIG. 3. Validation of the microarray results by QRT-PCR of the expression of a subset of representative transcripts whose abundance was found to be up-regulated by $P_4$ as assessed by cDNA microarray analysis. Gene-specific primers used are described in supplemental Table 2. The y-axis shows the amplitude of up-regulation determined by microarray (*blue bar*) or QRT-PCR (*red bar*) for the transcripts of the genes shown on the x-axis. Data shown are the mean ± SE of three experiments using independently isolated RNA preparations from the uterine epithelium as described in *Materials and Methods*.

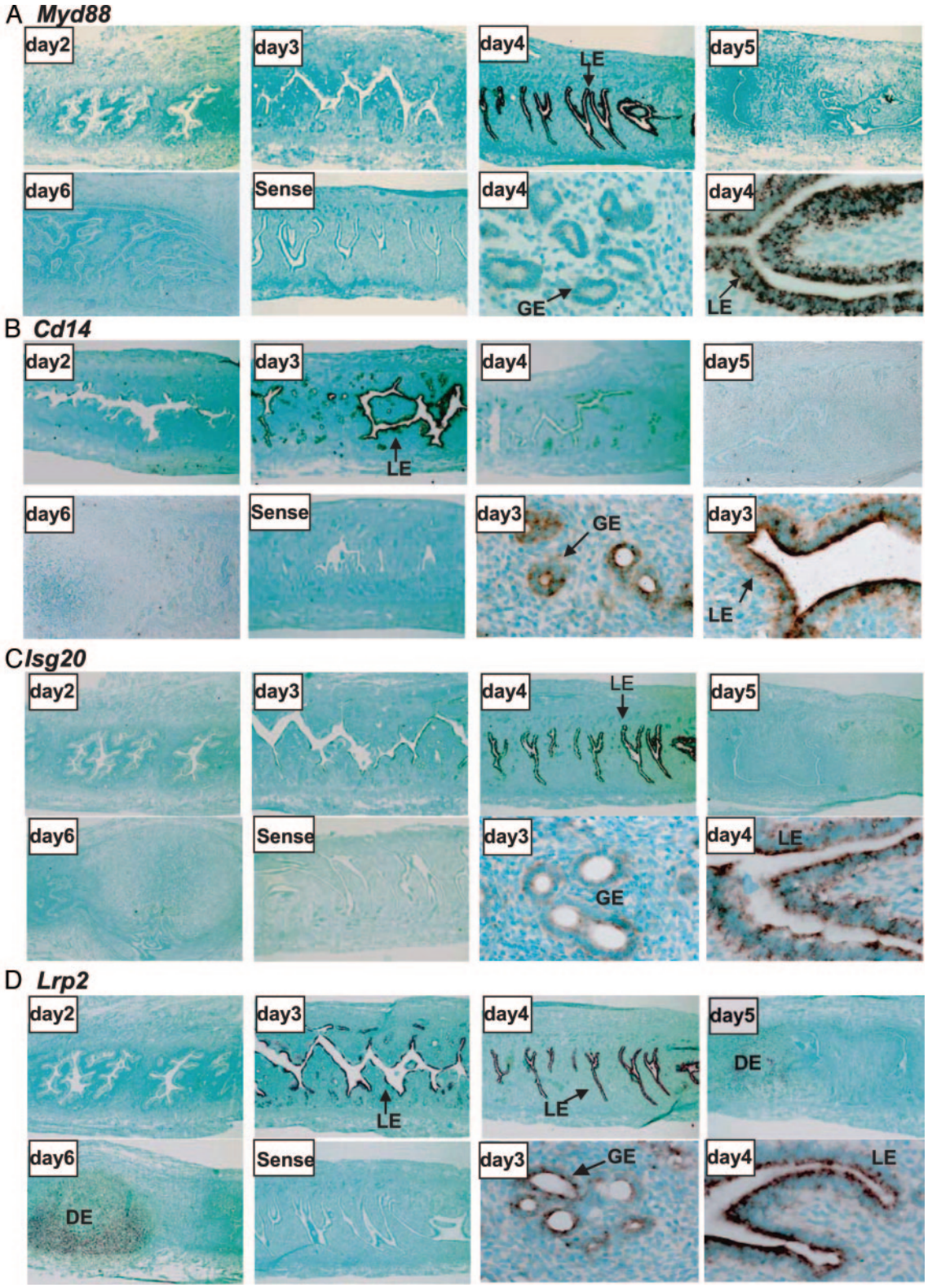


FIG. 4. *In situ* hybidization for a selected group of genes whose transcript abundance is up-regulated by $P_4E_2$. *In situ* hybridization of longitudinal uterine sections (×2.5) from d 2, 3, 4, 5, and 6 of pregnancy as shown using antisense probes for *Myd88* (A), *Cd14* (B), *Isg20* (C), and *Lrp2* (D). A representative section probed with sense probes is also shown in each panel. High-power (×40) micrographs are also shown of the luminal and glandular epithelium at the days indicated for each gene. For each gene all sections were hybridized and developed for the same time, and thus, relative levels between days are indicated by the intensity of the *purple-blue* precipitate that represents specific signal. However, cross-comparisons between genes expression levels are not possible using this method. Sections were counterstained with methyl green. LE, Luminal epithelium; GE, glandular epithelium; DE, decidua.

creased. For example, brain acyl-CoA hydrolase (*Bach-pending*) transcripts, encoding a cytosolic enzyme that cleaves acyl-CoA to free fatty acids and CoA-SH and known to be expressed in the brain and localized in neurons (43), are highly elevated in the uterine epithelium by $P_4E_2$. To further validate the microarrays and determine its expression pattern during the pre- and periimplantation period, we performed a Northern analysis of RNA isolated from the uterine luminal epithelium from pregnancy d 2 through 6 for *Bach*. Day 4 would be comparable with the period that we isolated RNA under this endogenous $P_4E_2$ hormone regimen (the nidatory $E_2$ being synthesized on d 4 of pregnancy). This Northern experiment showed a dramatic increase in *Bach* mRNA expression between d 3 and 4 of pregnancy (Fig. 5A). This expression was rapidly lost thereafter and was completely gone by d 5 and 6 (Fig. 5A). To confirm the *Bach* mRNA expression is in the uterine epithelium under physiological conditions, we also performed *in situ* hybridization with *Bach* antisense cRNA probes of uterine sections isolated from d 3 to 5 of a natural pregnancy. Expression of *Bach* mRNA was strongly detected in both the luminal and glandular epithelium on d 3 and 4 of pregnancy but was lost by d 5 (Fig. 5B). Interestingly *Bach* mRNA was also strongly expressed in the blastocyst captured in this section (Fig. 5B). No signal was detected using *Bach* sense probes (Fig. 5B).

Taken together, the specificity of expression in the luminal epithelium of all these genes and the coincidence of this expression with implantation validate our approach of isolating a single cell type isolated from uteri treated with precise hormonal regimens that mimic the physiological concentrations to identify differentially expressed genes in the luminal epithelium during the implantation window.

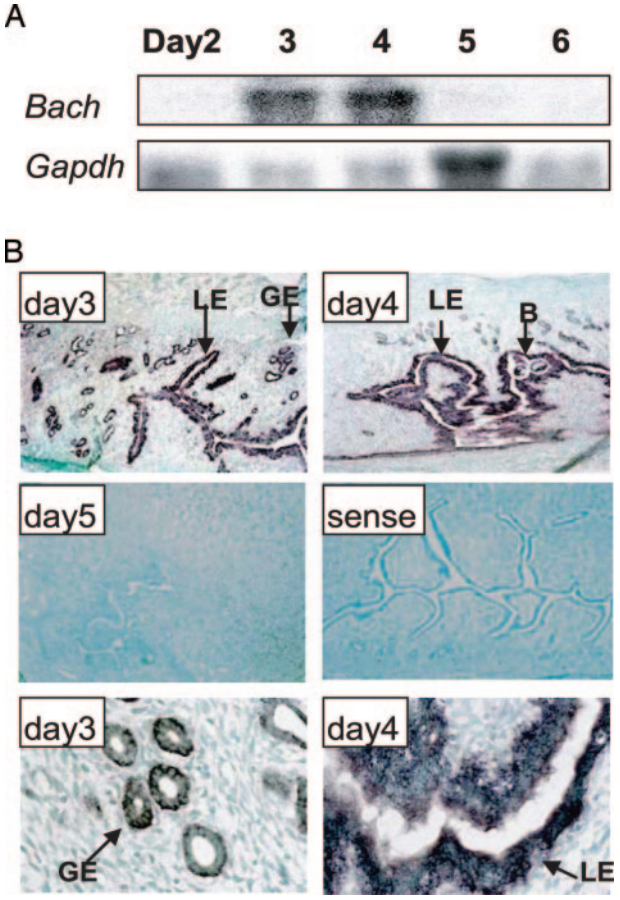


FIG. 5. Expression of brain acyl-CoA hydrolase (*Bach*) mRNA in mouse uterus during the pre- and periimplantation periods. A, Autoradiograph of a Northern analysis of *Bach* mRNA expression in RNA isolated from the uterine epithelium from d 2 to 6 of pregnancy. *Gapdh* mRNA acts as a loading control for the blots. B, *In situ* hybridization of uterine sections (×2.5) from d 3 to 5 of pregnancy using cRNA antisense probes to *Bach* mRNA. A sense cRNA probe was used on a d 4 pregnant uterine section as indicated. *Lower two panels* are high-power (×40) micrographs of the sections shown above. *Purple blue* precipitate represents positive hybridization. LE, Luminal epithelium; GE, glandular epithelium; B, blastocyst.

## Discussion

The sex steroid hormones $E_2$ and $P_4$ are the central regulators of the uterine preparation for implantation. Indeed the actions of these hormones alone support the differentiation of the uterus and the induction of decidualization in response to a drop of oil instilled into the uterine lumen, even in the absence of an embryo (44). Thus, signals from the blastocyst are not required for the early uterine events associated with pregnancy, although they may have a physiological role during normal pregnancy (45). The uterus is generally hostile to the implanting embryo, and it takes at least 2 d of $P_4$-regulated differentiation to prepare for $E_2$ to induce the receptive state (2). This state lasts for only about 24 h and is generally considered to involve a transient change in the uterine epithelium that allows blastocyst attachment and its subsequent invasion (46).

$E_2$ and $P_4$ exert their actions at the top of this hierarchy through ligand-activated receptors whose major functions appear to be transcription factors (40, 47). Thus, there have been several experiments aimed at defining the genes whose expression is altered in the uterus during the implantation window in both humans and mice. In mice these studies have used whole uterine tissue isolated from implantation, compared with interimplantation sites (19), implantation opposed to preimplantation stages (19, 48), progesterone receptor-deficient compared with wild-type mice (20), or antiprogestin RU-486-treated *vs.* untreated periimplantation stage mice (17, 40). However, the heterogeneous cell types of uterus that differentially respond to hormonal regulation make these array analysis difficult to interpret and many epithelial-specific genes may not be identified due to the small proportion of the epithelial cell type (~5%) in the whole uterus. Thus, in the present study, we used a method to isolate uterine epithelium from the remainder of the uteri to compare gene expression patterns in the uterine epithelium of mice treated with $P_4E_2$ and $E_2$.

This epithelial preparation has been shown to be more than 95% pure and largely to consist of luminal epithelia. This was shown by histological analysis before and after purification and by studies in which uterine epithelial or stromal nuclei were labeled with $^3$H-thymidine *in vivo* before isolation and the use of autoradiography to determine the percentage of nuclei labeled in each preparation, compared with the percentage of labeled nuclei in sections from the same uteri fixed before epithelial isolation (25). In addition,

biochemical analysis of cyclin A and nuclear cyclin D1 expression in the isolated fraction, compared with immunohistochemical detection on tissue sections, showed the same level of purity (24, 49). Furthermore, sets of genes whose expression is increased by $P_4E_2$ in the present study when analyzed by *in situ* hybridization showed specific expression in the uterine epithelia, validating that this approach identified genes expressed in the epithelial compartment.

Our DNA microarray analysis yielded 222 genes whose transcripts increase in abundance in the $P_4E_2$ group that could be classified using GO programs into 11 categories based on their relative biological functions. Consistent with the experimental design, several of these genes had already been identified as being regulated by $P_4$ or expressed during the implantation window. However, we also identified some genes whose expression had not previously been described during this period and whose transcript abundance is dramatically up-regulated in the luminal and/or glandular epithelium as demonstrated by our *in situ* hybridization experiments. These data provide overall validation of our approach that concentrated on the isolation of the epithelium and suggest that these genes may have specific and essential roles during implantation.

The implantation process has components of a classical proinflammatory response. This includes edema and the up-regulation of some inflammatory genes, such as *Cox-2*, that are induced solely in the stroma at the site of blastocyst attachment (50). However, the blastocyst is in most cases allogeneic to the mother, and thus, there must be mechanisms in place that suppress immune responses to the embryo (51). It is noticeable that there are dramatic changes in the influx of immune cells into the uterus that accompanies the expression of many inflammatory and hematopoietic cytokines (52). For example, at mating there is an influx of macrophages and eosinophils recruited by their sex steroid hormone-regulated growth factors and cytokines, colony-stimulating factor (CSF)-1 and eotaxin, respectively (22, 53). However, this inflammatory response on d 1 of pregnancy is transient. For example, Northern blot analysis showed that IL-1$\alpha$, IL-1$\beta$, and TNF$\alpha$ mRNA, all cytokines involved in a classical inflammatory response, are induced on d 1 of pregnancy, but their expression is significantly reduced on d 2 and maintained a basal level on d 3 and 4 of pregnancy (54, 55). However, TNF$\alpha$ and IL-1$\alpha$ mRNA and protein are still found in both human and mouse uterine epithelium at implantation (54, 56). Indeed, there is some controversial evidence that IL-1$\alpha$ is required for implantation (57, 58). DNA microarray analyses have also documented a set of immune-related genes that are down-regulated at the implantation sites (19). A large proportion of these genes encoded the classical immunoglobulins. This suggests that B cells are excluded from the implantation site, suggesting one mechanism whereby the embryo may escape the maternal immune response. However, none of these genes are represented in the current genes list. This is most likely because the other array experiments used whole uterine tissues, and the lymphocytes that would express these Ig genes are mostly found in the stroma.

It is noticeable that in our and other studies, that many genes whose expression is associated with the innate immune response are found in the cohort with increased transcript abundance. Some of these appear to be involved in responses to interferon, such as *Isg20* described in the current study or interferon-response gene 1 described by others (59). Constitutive expression of ISG20 conferred resistance to vesicular stomatitis virus and influenza virus infection in HeLa cells even without interferon-$\gamma$ treatment (38). This gene (previously known as HEM45) was also identified by differential display experiments of whole rat uteri as an $E_2$-responsive gene (60). We did not find this in the $E_2$-regulated class, but our comparisons were restricted to epithelia and did not analyze the induction of genes by $E_2$ in comparison with ovariectomized mice. Thus, *Isg20* maybe also be regulated by $E_2$ and superregulated by $P_4E_2$. Others genes in this group include those that encoding the mononuclear phagocytic growth factor, CSF-1, whose transcript and protein abundance is increased by $E_2$ and $P_4$ acting synergistically in the uterine epithelium just before implantation (8). This hormonally induced CSF-1 regulates the uterine acquisition of macrophages as well as the innate immune response in the face of infection through its action on cells of trophectodermal origin (22, 61). Indeed, the uterine epithelium is a major producer of hematopoietic cytokines through the periimplantation period, suggesting that these play a major role in orchestrating immunity at the uteroembryonic interface (62, 63).

Several of these immune response cytokines such as TNF$\alpha$ and IL-I$\alpha$ are downstream of NF-$\kappa$B signaling. It was noticeable in our studies that components of the TLR4 pathway that stimulates NF-$\kappa$B signaling were dramatically up-regulated in the luminal epithelium during this periimplantation period. These included mRNAs for the TLR4 receptor cofactor CD14, the adapter protein MYD88, and downstream genes such as *Irg1*. Of these, both *Myd88* and *Irg1* were also identified in array experiments comparing progesterone receptor knockout (PRKO) mice with wild-type periimplantation mice (20). *Irg1* mRNA is also elevated in macrophages by LPS whose action is mediated through TLR-4 and the protein kinase C pathway (64). We had previously identified this gene's transcripts as being very significantly increased in the luminal epithelium by $P_4$ and $E_2$ and also through a protein kinase C-mediated pathway, suggesting that $P_4$ may in some way activate the TLR4 pathway (14). Antisense oligonucleotides directed against *Irg1* mRNA, when instilled intraluminally into the uterus, inhibited implantation, suggesting that this gene plays an important role in this process (15). The up-regulation of components of the TLR4 pathway as well as expression of genes downstream of NF-$\kappa$B such as *Irg1*, *Tnf$\alpha$*, and *IL-I$\alpha$* suggests that the NF-$\kappa$B signaling pathway becomes activated in the uterine luminal epithelium at implantation. This contention is supported by EMSA of whole uterine homogenates that indicated NF-$\kappa$B activation during the implantation window (65). Furthermore, exogenous expression of inhibitory-$\kappa$B$\alpha$, an inhibitor of NF-$\kappa$B signaling in the uterus after intraluminal infection with a Sendai viral vector, caused a modest delay in the timing of implantation, suggesting an effect on the window of receptivity (66). These data together suggest that NF-$\kappa$B signaling is an important component of the regulation of blastocyst implantation.

The TLRs are part of a pathogen recognition system (37). However, in the sterile LPS-free environment of the mouse, it is interesting to speculate that there might be a natural ligand for these receptors. In contrast, infection of the uterus by intraluminal administration of *Listeria monocytogenes* that engages TLR2 and also signals through Myd88 is sufficient to induce decidualization in hormonally primed uteri, further suggesting an important role for NF-κB in this pathway (67). The presence of this signaling system also suggests that the uterus is primed to respond to pathogens that might have been introduced into the reproductive tract as a result of mating. These might prematurely activate this pathway, causing asynchrony in the uterine response, and this may be the reason why infectious agents are a major cause of early pregnancy loss.

Sex steroid hormone action has long been considered to be the result of sequential activation of gene batteries cascading downstream of the original receptor occupancy. It is noticeable, therefore, that there were several transcription factors whose transcripts were up-regulated by $P_4E_2$ treatment. Noticeable among them are *Bteb1* (krüppel-like factor, *Klf9*) and *Pnrc2* that interact with steroid hormone receptors and are regulated by or regulate nuclear factor-Y (NF-Y), a key controller of cell cycle gene expression (68–71). Importantly, gene ablation of *Klf9* resulted in an implantation defect and also a perturbation of hormone-regulated uterine luminal and glandular epithelial cell proliferation that may be the cause of implantation defect (72, 73). We have demonstrated that $P_4E_2$ treatment coordinately down-regulated the transcripts of 20 genes associated with DNA replication licensing, DNA synthesis, and nucleosome modifications in the uterine luminal epithelium (Pan, H., Y. Deng, and J. W. Pollard, unpublished observations). The rapid up-regulation of a cohort of transcriptional regulators, some of which are known to be involved in cell cycle gene regulation, suggests that these could be responsible for the coordinate down-regulation of these DNA replication and licensing genes.

It has been assumed that the intimate cross-talk between the blastocyst and uterus during the attachment reaction share features of the reciprocal heterotypic cellular interactions found during embryogenesis. A growing body of evidence shows that many conserved signaling pathways that determine pattern formation in the embryo development are also implicated in the implantation process. These include members of the hedgehog, bone morphogenic (Bmp), Wnt, and Homeobox gene families (40, 41, 74, 75). Interestingly, in our microarray screen, we identified another important developmental gene, low-density lipoprotein receptor-related protein (*Lrp-2*). Its expression is specifically limited in the uterine epithelium coincident with the implantation window. *Lrp-2* belongs to low-density lipoprotein receptor gene family that is involved in lipoprotein metabolism. During development *Lrp-2* deficiency resulted in an increase of dorsal signaling through BMP4 expression and subsequent loss of sonic hedgehog (*Shh*) expression in the ventral forebrain. It has been proposed that LRP-2 acts as a BMP4 clearance receptor that mediates endocytic uptake and degradation of BMP4 (42). Fascinatingly, *Lrp-2*, whose other name is Magellin, has recently been found to be essential for the transport of sex steroid hormones into cells, and its loss results in developmental abnormalities of the male and female reproductive tract (76). Its expression at implantation suggests that there may be a similar role in sex steroid hormone transport at implantation.

The array data presented here has revealed many genes whose transcripts are increased in abundance in the epithelium during the implantation window, and *in situ* hybridization has revealed at least seven expressed in the luminal epithelium. The data suggest new pathways that may be involved in implantation and adds links to other data that have shown necessity for some signaling pathways during implantation. Indeed, in our related study (21) whereby we analyzed gene expression patterns between luminal and glandular uterine epithelia isolated by laser capture microdissection from tissue obtained 8 h after the nidatory $E_2$, there was coincidence between the two studies in detection of genes such as *Irg1*, *Sultn*, *Gsto1*, and *Cnn3*. However, there are also some that were not found in this study that would correspond to 3-h postnidatory $E_2$. This suggests a dynamic pattern of gene expression through the periimplantion period. In addition, to those genes whose identity is known, there are also many whose identity is unknown (expressed sequence tags or Rikagaku Kenkyusho cDNAs). Studies looking at the kinetics of patterns of gene expression in response to these steroid hormones by cluster analysis may allow placement into groups of genes with known functions of some of these unknown genes, data that may warrant their further study. In the meantime, to define the functions for the many known genes in the implantation process, there is a requirement for the development of tools to specifically interfere with their function in the luminal epithelial cells in an acute way at implantation.

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