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Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation

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Tierney, Emily P., Suzana Tulac, Se-Te Joseph Huang, and Linda C. Giudice. Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation. *Physiol Genomics* 16: 47–66, 2003. First published October 7, 2003; 10.1152/physiolgenomics.00066.2003.—Decidualization of endometrial stromal cells is a prerequisite for human implantation and occurs in vivo in response to progesterone and involves activation of the protein kinase A (PKA) pathway. The objective of this study was to determine the molecular signatures and patterns of gene expression during stimulation of this pathway with an analog of cAMP. Endometrial stromal cells from two subjects were treated with or without 8-Br-cAMP (1 mM) for 0, 2, 12, 24, 36, and 48 h and were processed for microarray analysis, screening for 12,686 genes and ESTs. Most abundantly upregulated genes included neuropeptides, immune genes, IGF family members, cell cycle regulators, extracellular matrix proteases, cholesterol trafficking, cell growth and differentiation, hormone signaling, and signal transduction. Most abundantly downregulated genes included activator of NF-κB, actin/tropomyosin/calmodulin binding protein, cyclin B, IGFBP-5, α1 type XVI collagen, lipocortin III, L-kynurenine hydrolase, frizzle-related protein, and cyclin E2. RT-PCR validated upregulation of IGFBP-1, preprosomatostatin, and IL-11, and Northern analysis validated their kinetic upregulation. RT-PCR confirmed downregulation of IGFBP-5, cyclin B, and TIL-4. K-means analysis revealed four major patterns of up- and downregulated genes, and genes within each ontological group were categorized into these four kinetic patterns. Within each ontological group different patterns of temporal gene expression were observed, indicating that even genes within one functional category are regulated differently during activation of the PKA pathway in human endometrial stromal cells. Overall, the data demonstrate kinetic reprogramming of genes within specific functional groups and changes in genes associated with nucleic acid binding, cell proliferation, decreased G protein signaling, increased STAT pathway signaling, structural proteins, cellular differentiation, and secretory processes. These changes are consistent with cAMP modulating early events (0–6 h) primarily involving cell cycle regulation, subsequent events (12–24 h) involving cellular differentiation (including changes in morphology and secretory phenotype), and late events (24–48 h) mediating more specialized function, including immune modulators, in the human endometrial stromal cell.

endometrium; decidualization; implantation; microarray technology

DURING NORMAL, OVULATORY MENSTRUAL cycles, human endometrial stromal cells undergo the differentiative process of "decidualization" in which they adopt a unique morphologic, biosynthetic, and secretory phenotype (24). When pregnancy ensues, the endometrial stromal compartment becomes uniformly decidualized and comprises the decidua, a morphologically and functionally distinct tissue that persists throughout gestation and represents the maternal aspect of the maternal-fetal interface (24). Decidualization in vivo involves progesterone and the cAMP pathway. Decidualization in vitro has been shown to be mediated by progesterone, after estradiol (E2) priming, as well as by cAMP and other activators of the protein kinase A (PKA) pathway (6, 12, 53, 54, 60). Early in the process of decidualization, endometrial stromal cell mitosis occurs, accompanied by endoreduplication of DNA (54). Marked changes occur in cytoskeletal organization and cell adhesion, as well as in the extracellular matrix, and there is a unique set of secreted proteins and peptides. Decidualized endometrial stromal cells synthesize and secrete "markers" of decidualization, including prolactin and IGFBP-1, and produce extracellular matrix components typical of gestational decidual cells, including laminin, type IV collagen, fibronectin, and heparin sulfate proteoglycan (for review, see Ref. 24). However, the program of gene expression changes and, thus, mechanisms underlying development of the decidualized phenotype are not well defined.

Decidualization involves transformation of endometrial spindle-like fibroblasts into polygonal epithelial-like cells that are hypothesized to regulate placental trophoblast invasion into the endometrium during the invasive phase of implantation (53). Stromal cells undergo these characteristic morphologic changes when treated with E2/P4 and/or cAMP in vitro (6, 39, 53, 54).

Decidualization is known to be mediated by the PKA pathway (24, 54, 60) and relaxin, which acutely and permanently elevates cAMP levels, and induces prolactin expression and the morphologic changes of decidualization in human endometrial stromal cells. Also, PKA pathway members including regulatory subunit isoforms (RIα, RIβ, RIIα, RIIβ) and catalytic subunits (Cα and Cβ) are upregulated in stromal cells treated with relaxin (54). In progesterone-induced decidualization, progesterone acts through the PKA pathway, elevating both prolactin and intracellular cAMP levels (6). The PKA inhibitor, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, significantly suppresses progesterone-dependent prolactin expression (6). In addition, cAMP is an independent mediator of decidualization (39, 53). cAMP derivatives promote the differentiation of the fibroblast-like stromal cells to the decidual phenotype and induce the expression of products characteristic of decidual cells, e.g., prolactin, IGFBP-1, desmin, hsp 27, and laminin (6, 39, 53).

Microarray analysis has been used to investigate gene expression in in vitro models of decidualization in endometrial stromal cells (39) and term decidual fibroblasts (49). In our

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initial cDNA microarray study with human endometrial stromal cells from nonpregnant subjects, comparison was made of genes upregulated at fixed time points, i.e., after 48 h in response to 8-Br-cAMP and after 10 days of treatment with estradiol (E2) and progesterone (39). Numerous gene families were upregulated with treatment with both cAMP and E2/P4, with concordance between the two different treatment groups, and included growth factors, neuromodulators, inflammatory cytokines, cell adhesion molecules, oncogenes, and transcription factors. A recent study on human term pregnancy decidual fibroblasts treated for 15 days with E2/P4 and cAMP revealed dynamically regulated genes with reprogramming of gene expression within functional categories, suggesting fundamental aspects of cellular differentiation (6).

Herein, we present the results of high-density oligonucleotide microarray analysis and K-means kinetic pattern grouping of genes and gene families expressed in nonpregnant human endometrial stromal cells during the first 48 h of treatment with 8-Br-cAMP. cAMP was utilized, as it is known to play a role during in vitro decidualization of the human endometrial stromal cell. Although cAMP may not be the exclusive mediator of decidualization in the human endometrial stromal cell in vitro, it has been shown to be a potential key mediator in the decidualization process as levels of PKA pathway intermediates increase with decidualization and inhibitors of the PKA pathway result in decreased expression of markers of decidualization, i.e., prolactin, IGFBP-1. The results of our array reveal genes previously known to be induced or downregulated in response to cAMP, as well as numerous newly recognized genes and gene families. In addition, up- and downregulation of genes within families and within specific functional groups supports reprogramming as an important mechanism in activation of the PKA pathway in human endometrial stromal cells.

MATERIALS AND METHODS

Cell Culture

Endometrial samples were obtained from two different subjects, after written informed consent and using a protocol approved by the Stanford University Committee on the Use of Human Subjects in Medical Research. Subjects were 36- and 39-yr-old Caucasian females and underwent hysterectomy for leiomyomas. Tissues from both patients were obtained during the secretory phase of the menstrual cycle. Stromal cells were isolated by enzymatic digestion and cultured as described (25). Endometrial tissue was digested with collagenase, and stromal cells were isolated and plated with DMEM/MCDB-105 medium containing 10% charcoal-stripped fetal bovine serum (FBS), insulin (5 μg/ml), gentamicin, penicillin, and streptomycin. At the fourth passage, cells were plated at a density of 6×10^5 cells/10 cm^2 for treatment with cAMP in serum-free medium. After cells reached confluence, plates were treated with 1 mM 8-Br-cAMP (in serum-free medium) for a time course of 48 h. All cells were cultured in serum-free medium (DMEM/MCDB-105 medium containing ascorbic acid, transferrin, and gentamicin) for 2 days prior to the onset of decidualization treatment. After 24 h of decidualization with cAMP, it was noted that the cells began a transformation from elongated spindle-like fibroblasts into polygonal epithelial-like cells. At 48 h, a predominance of the cells treated with cAMP had transformed into polygonal epithelial-like cells, characteristic of decidualized cell morphology.

At each time point (0, 2, 12, 24, 36, and 48 h), medium was collected for analysis of IGFBP-1 levels by ELISA (Diagnostic Systems Labs, Webster, TX), and TRIzol was added immediately to cells to isolate total RNA. Patient samples utilized for the microarray analysis were selected based upon their similar levels of high peak concentrations of secreted IGFBP-1 at all time points, as measured by ELISA (see Fig. 2 of RESULTS).

Gene Expression Profiling

At 0-h, 2-h, 12-h, 24-h, 36-h, and 48-h samples were processed individually for microarray hybridization following the Affymetrix protocol (Affymetrix, Santa Clara, CA). Total RNA yield was 30–40 μg. Total RNA was purified via RNeasy RNA purification (Qiagen) according to the manufacturer's instructions. Purity of the RNA was assessed spectrophotometrically by the $A_{260/280}$ ratio. A T7-$(dT)_{24}$ oligo-primer was used for double-stranded cDNA synthesis by the SuperScript Choice System (GIBCO-BRL). In vitro transcription was subsequently conducted with Enzo BioArray High Yield RNA T7 transcript labeling kits (Enzo, Farmingdale, NY) Additional cRNA cleanup was performed using RNeasy spin columns (Qiagen), prior to chemical fragmentation with 5× fragmentation buffer (200 mM Tris, pH 8.1, 500 mM KOAc, 150 mM MgOAc). After chemical fragmentation, biotinylated cRNAs were hybridized to Affymetrix GeneChip Hu95A oligonucleotide microarrays on an Affymetrix fluidics station at the Stanford University School of Medicine Protein and Nucleic Acid (PAN) Facility. Fluorescent labeling and laser confocal scanning were conducted in the PAN facility and generated the data for analysis.

Validation of Gene Expression Data

RT-PCR. Total RNA was used in a reverse transcriptase reaction (1 μg RNA/20 μl reaction volume, 10× RT buffer, dNTPs, oligo-dT, RT enzyme) which was subsequently utilized in a PCR reaction (RT, 10× *Taq* buffer, $MgCl_2$, dNTP, *Taq* enzyme) with primers (Table 1) specific for preprosomatostatin, IGFBP-1, IL-11, cyclin B, TIL-4, IGFBP-5, and GAPDH. PCR reaction conditions were 34 cycles of the following sequence: 3 min at 94°C, 45 s at 94°C, 45 s at 56°C, 45 s at 72°C, followed by 10 min at 72°C with subsequent cooling to 4°C.

Northern blots. Endometrial samples from two subjects were utilized for the Northern blots. One of the samples was from the same

Table 1. *Oligonucleotide primers with predicted respective PCR product sizes*

Gene	Forward Primer	Reverse Primer	bp
GAPDH	cacagtccatgccatcactgc	ggtctacatggcaactgtgag	609
GAPDH	accacagtccatgccatc	tccaccaccctgttgctg	452
IGFBP-1	actctgctggtgcgtctac	ttaaccgtcctccttaaac	499
IL-11	gcacagctgagggacaaatt	ccagtcaagtgtcaggtgca	390
Preprosomatostatin	aatcgaagggtctcgctgaag	gctgtcataccgcctccagt	450
IGFBP-5	ggaattcgaagcagtgaagaaggaccg	actcaacgttgctgctgtcgaaggtgt	376
Cyclin B	attgactggctagtacaggt	gtagagttggtgtccattca	480
TIL-4	cacagaagctgtaaaaagcc	ccacggaacttgtaacatct	524

Forward and reverse primer sequencer used for RT-PCR for microarray validation, with expected PCR product sizes.

patient as utilized in the microarray experiment, and the other displayed similar levels of IGFBP-1 (1,500–2,000 ng/ml) at 48 h as the samples utilized in the microarray. Total RNA was isolated using TRIzol at time intervals of 0 h, 6 h, 12 h, 24 h, and 48 h. Total RNA (20 μg) was electrophoresed on 1% formaldehyde agarose gel and transferred to a nylon membrane for Northern analyses. Specific ^{32}P-labeled cDNA probes for GAPDH, IGFBP-1, IL-11, and preprosomatostatin, ranging from 400–600 bp, were generated using Ready-to-Go random primer kit (Pharmacia Biotech, Peapack, NJ) and [^{32}P]dCTP (NEN Life Science Products, Boston, MA). The membrane was prehybridized at 68°C for 30 min in ExpressHyb buffer (Clontech, Palo Alto, CA), and hybridization was carried out for another hour at 68°C using ExpressHyb buffer containing 1–2 × 10^6 cpm/ml of labeled probe. Washing was subsequently carried out according to the manufacturer's instructions. Membranes were exposed to Kodak MS X-ray films, and densitometry was performed with an imaging densitometer (model GS-710; Bio-Rad, Hercules, CA) and analyzed by its accompanied software Quantity One, ver. 4.0.2. GAPDH mRNA intensities were used for normalization prior to comparison. Stripping and reprobing were performed to utilize the same membrane for all four genes studied.

Data Analysis

Data were analyzed with GeneChip Analysis Suite ver. 4.01 (Affymetrix), GeneSpring ver. 4.21 (Silicon Genetics, Redwood City, CA), and Microsoft Excel 2001 software. Expression profile data was first prepared using GeneChip Microarray Analysis Suite and subsequently exported to GeneSpring for further analysis. Within each hybridization, the 50th percentile of all measurements was used as a positive control, and the measurement for each gene was divided by this control. The bottom 10th percentile was used for background subtraction. Between different hybridization outputs, each gene was normalized to itself by making a synthetic positive control for that gene comprised of the median of the gene's expression values over all samples in an experimental group and dividing the measurements for that gene by the positive control, as specified in the manufacturer's instructions. Mean values were then calculated for each gene probe set among individual time points, and the fold change difference between expression at a particular time point and the expression at the time point of 0 h was calculated for each cell line studied. Data are reported in RESULTS (see Supplemental Tables A and B, available at the *Physiological Genomics* web site)[1] for the specific genes upregulated and downregulated at individual time points, where the fold change is calculated as the ratio of the gene expression between an individual time point and the gene expression at 0 h.

K-means and Ontology Analysis

Genes were selected for consideration for K-means and ontological analysis based upon their apparent expression change relative to the *time 0* reference sample, where only genes demonstrating a twofold or greater change in expression at a particular time point were utilized for the analysis. This approach of only analyzing trends of the most highly regulated genes, as utilized in Aronow et al. (1), allowed detection of coordinately regulated groups of genes during the time course without the dilution effect of genes whose expression did not change significantly during treatment with cAMP. For the ontology analysis, genes demonstrating a twofold or greater change at each individual time point were utilized for our analysis. Ontological groupings were classified into biological function groupings based upon the categories listed in the GeneSpring program (Silicon Genetics). K-means analysis and tree diagrams in the GeneSpring program (Silicon Genetics) were utilized to analyze the temporal pattern of gene expression within each ontology category of gene function. For the K-means analysis of the temporal trends in gene expression, genes with regulation greater than twofold in both samples in half or more of the time points were included in the analysis. K-means was applied to the data using standard correlation analysis. Genes were clustered according to their expression pattern dynamics by subjecting the log2-transformed data set [$R = \log2(x_{t=i}/x_{time\ o})$], where R is the expression ratio for each gene, to the K-means as implemented in the GeneSpring program (Silicon Genetics). Four groups of genes with different characteristic temporal expression patterns were derived from the K-means analysis of up- and downregulated genes. Four groups were utilized to categorize the individual kinetic patterns for both the up- and downregulated genes, as a smaller number of groups did not adequately distinguish gene behavior and a larger number of groups did not display significant distinguishable trends between groups. Overall trends of temporal gene expression in the comprehensive data set were compared with those in the individual ontological groupings using K-means analysis to identify trends in gene expression by functional classification during the onset of treatment with cAMP.

Microarray data for this manuscript are available on line at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (**http://www.ncbi.nlm.nih.gov/geo**), with accession number GSE403.

RESULTS

Fold Up- and Downregulation in Response to cAMP

A sequential pattern of gene induction or repression was observed in cultured endometrial stromal cells in response to 8-Br-cAMP. Figure 1, *A* and *B*, graphically displays the trend of gene expression relative to the expression at the 0 h time point, during the time course studied. The ontological groupings for the genes expressed at a level twofold and higher, relative to the 0 h nondecidualized control, and their levels of expression at 2, 12, 24, 36, and 48 h are presented in Supplemental Table A. Supplemental Table B lists the downregulated genes at these same time points. Levels of IGFBP-1 secreted by the individual patient samples used in the microarray are graphically displayed in Fig. 2.

Gene Ontology

Some of the most markedly upregulated genes (Table 2*A*) include neuropeptides, immune genes, members of the IGF family, extracellular matrix proteases, genes involved in cell growth and differentiation, hormone signaling, NF-κB protein kinase cascade, GABA R signaling, coagulation, and early steroid hormone responsiveness. Some of the most markedly downregulated genes (Table 2*B*) include activator of NF-κB, actin/tropomyosin/calmodulin binding protein, cyclin B, IGFBP-5, L-kynurenine hydrolase, cyclin B2, and cyclin E2.

Gene Clustering by Temporal Expression Pattern

Many of the gene families under study exhibited interesting patterns of temporal gene expression, correlating with their proposed functional roles in human endometrium. Figures 3 and 4 demonstrate the results of the K-means analysis for the up- and downregulated genes, respectively, where the data were clustered into four groups of temporal expression patterns, based upon similarity of expression patterns, as measured by standard correlation (10). For the upregulated genes (Fig. 3) the first group of genes identified demonstrates low

[1] The Supplementary Material for this article (Tables A and B) is available online at **http://physiolgenomics.physiology.org/cgi/content/full/00066.2003/DC1**.

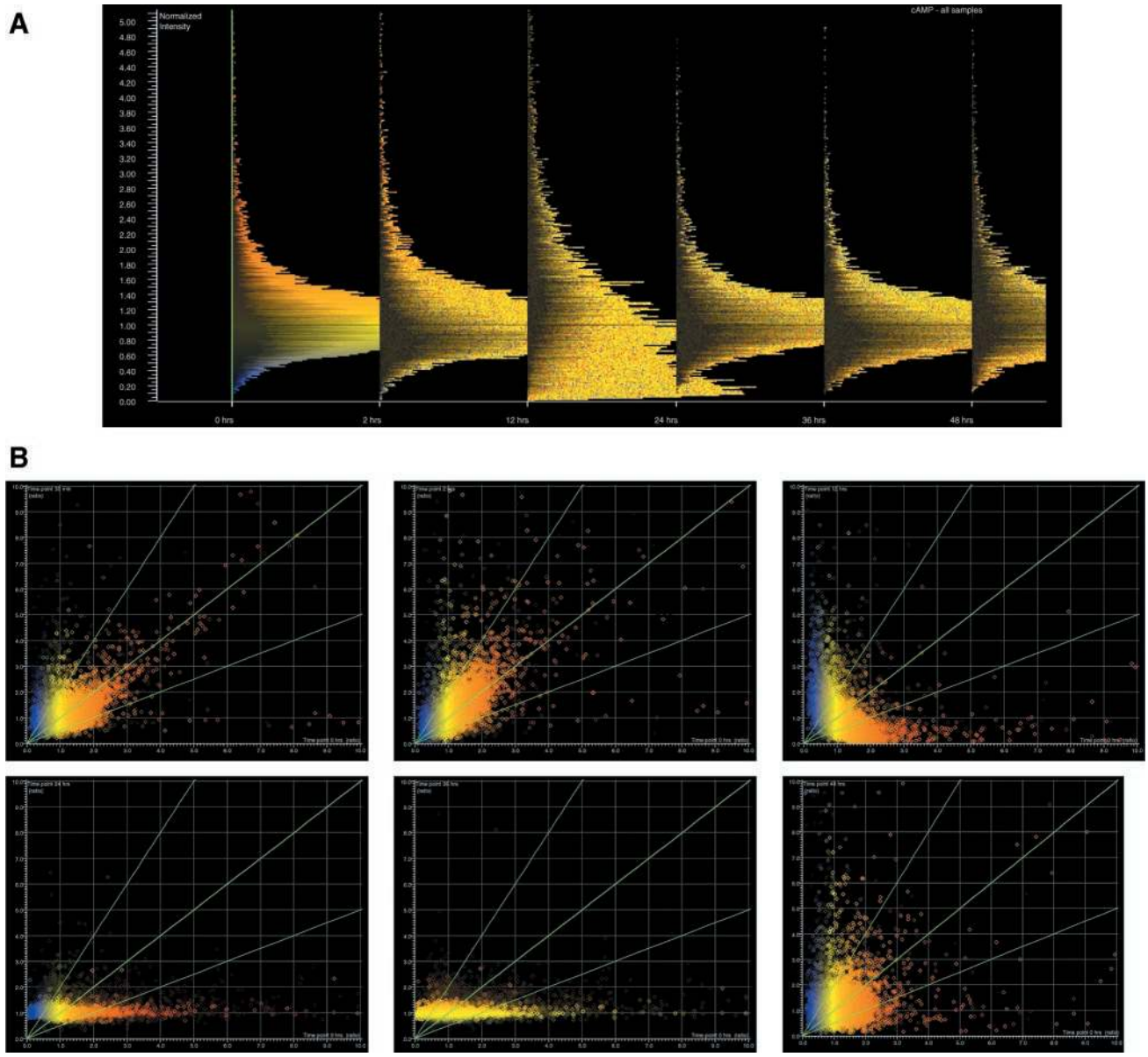


Fig. 1. Scatter plots of microarray data showing composite normalized data (*A*) analyzed at 6 different time points (0, 2, 12, 24, 36, and 48 h) during decidualization with 1 mM 8-Br-cAMP. Normalized relative intensity of each gene is represented on the vertical axis. Each individual time point (0, 2, 12, 24, 36, 48 h) is represented on the *x*-axis. *B*: demonstrates scatter plot of microarray data demonstrating pattern of expression at 0 h, 2 h, 12 h (*top* row, *left* to *right*), 24 h, 36 h, and 48 h (*bottom* row, *left* to *right*). Scatter plots demonstrate relative intensity of each gene at individual time points relative to the 0 h time point. All gene expression data shown reflect the relative expression after normalization. The *y*-axis demonstrates the normalized relative intensity of each gene at the particular time point, and the *x*-axis demonstrates the normalized relative intensity at the 0 h time point.

expression in the first 12 h of treatment with cAMP, with a sharp increase in expression between 12 and 24 h, and then levels sustained throughout the remainder of the 48 h time course. The second K-means group exhibits a biphasic pattern of peak gene expression at 12 h and 48 h. The third K-means group shows a peak expression at 12 h with a subsequent fall in expression for the remainder of the 48 h time course. The final pattern demonstrates a gradual increase in gene expression throughout the time course with a more pronounced increase in expression observed in the second half of the time course. The downregulated genes (Fig. 4) similarly illustrate four patterns of temporal expression using K-means analysis. The first group of genes exhibit sequential downfold induction throughout the entire time course. The second K-means group shows upregulation between 0 and 2 h and downfold induction at 12 h that persists until 48 h. The third group demonstrates a biphasic pattern of a decrease in expression at both 12 and 48 h. The final pattern of gene expression observed is upregulation from 0–12 h and downfold induction from 24 through 48 h.

Trends in Gene Expression by Ontological Group

Tables 3 and 4 demonstrate the number of genes in each ontological category that fit into each of the four K-means

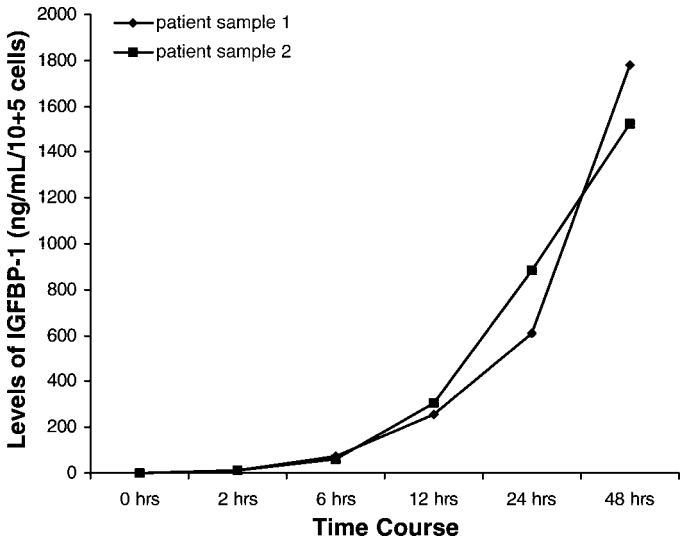


Fig. 2. IGFBP-1 levels (ng/ml per 10^5 cells) seen in endometrial stromal cells decidualized with 1 mM 8-Br-cAMP utilized in the microarray.

cluster patterns of temporal gene expression for the up- and downregulated genes, respectively. Temporal patterns of gene expression of the ontological categories (Figs. 5*A* and 6*A*) and select subcategories (Figs. 5*B* and 6*B*) of the up- and downregulated genes, respectively, are displayed graphically utilizing tree diagrams. Even genes within one functional categorization are induced or repressed along different timeframes and patterns during cAMP treatment. Many of these trends closely parallel the above four cluster groupings identified in the K-means analysis.

For the upregulated genes, a subset of the cell growth, enzymes, immune proteins, signal transduction cascades, and structural proteins exhibit *pattern 1*. A subset of all major ontological groupings demonstrates *pattern 2* of a biphasic pattern of peak expression at 12 and 48 h. Included in this group are members of the orphan steroid hormone receptors NOT and NGFI-B/nur77; however, these genes exhibit the first peak of increased expression at 2 h of treatment with cAMP, consistent with their known roles as immediate early response genes. Also in this group are cell cycle regulation, immune proteins, enzymes, nucleic acid binding proteins, and signal transduction. *Patterns 3* and *4* are observed in a subset of cell growth, cancer/cell cycle regulation, enzymes, nucleic acid binding, signal transduction, and structural proteins.

For the downregulated genes (Table 4), a number of cancer/cell cycle regulation, enzymes, G protein receptor signaling, and extracellular matrix proteins demonstrate *pattern 1*. A significant subset of all major ontological categories and subcategories of downregulated genes, including cell growth, cancer/cell cycle regulation, enzymes, immune proteins, signal transduction, structural proteins, and G protein receptor signaling, is represented in *pattern 2*. *Pattern 3* is predominantly seen in a significant subset of the cell growth, enzymes, and cancer/cell cycle regulation genes, and *pattern 4* includes a significant subset of cell death, immune proteins, cancer/cell cycle regulation, enzymes and signal transduction genes. These up- and downregulated genes are described in more detail below.

Ontological Trends of Upregulated Genes

Many of the cancer and cell cycle regulation genes follow *pattern 2*, with an earlier first peak at 2 h (e.g., N-ras) or more commonly, peak expression at 12 h and 48 h [e.g., Von Hippel-Lindau (VHL) gene and p126 (ST5)]. Other cell cycle regulatory genes, including NF1 and trk, demonstrate a more gradual progressive increase in expression during the time course studied. Some cell growth genes can be divided into two categories based upon their pattern of temporal expression. One group, including Rb107 and carcinoembryonic antigen, follows *pattern 3*, and another group shows low levels of initial expression and subsequently a rapid increase in between 12 and 24 h, reaching a plateau and remaining elevated thereafter. This latter group includes IGFBP-1, VEGF receptor-2, EBAF, and TGF-β1 binding protein, clone L5 orphan G-protein-coupled receptor, IGF-1 receptor, TGF-β2, and inhibin β_B-subunit.

The gene subfamily which signals through the STAT protein kinase cascade (including the neuropeptides) also demonstrates

Table 2. *Most markedly up- and downregulated genes during the time course of decidualization in response to 8-Br-cAMP*

Ontologic Category/ Gene Name	2 h	12 h	24 h	36 h	48 h
A: Upregulated genes					
Neuropeptides					
Preprosomatostatin	115.49	116.39	1644.4	1396.82	1324.2
L-orphan (clone 5) G protein coupled R	3.62	6.56	79.77	126.18	123.26
Immune genes					
IL-11	7.1	2.62	28.3	74.9	62.98
Factor XIII precursor	NA	NA	26.98	28.78	25.815
Cell growth/ differentiation					
Prolactin	NA	3.88	99.45	143.41	37.135
IGFBP-1	8.38	8.375	142.52	140.54	115.78
EBAF	NA	3.48	70.49	123.86	51.37
NGFI-B/nur77 transcr. factor	106.4	NA	17.23	14.11	30.12
NOT	350.94	2.24	24.73	30.19	113.14
Extracellular matrix protease					
MMP-10 (stromelysin-2)	4.25	2.13	148.48	144.2	69.71
B: Downregulated genes					
Cell growth					
IGFBP-5	NA	8.925	8.81	9.015	80.185
Enzymes					
L-Kynurenine hydrolase	7.61	NA	3.33	6.9	169.32
Signal transduction					
Activates NF-κ B	7.87	17.51	2.19	4.18	369.01
Structural protein					
Actin, tropomyosin, calmodulin binding protein	NA	22.32	13.17	12.59	50.21
Cell cycle					
Cyclin B2	NA	5.69	9.07	5.17	10.2
Cyclin E2	2.12	NA	NA	NA	9.28

Numbers expressed are fold change, relative to *time 0.* NA, genes with upregulation of less than 2-fold at a given time point.

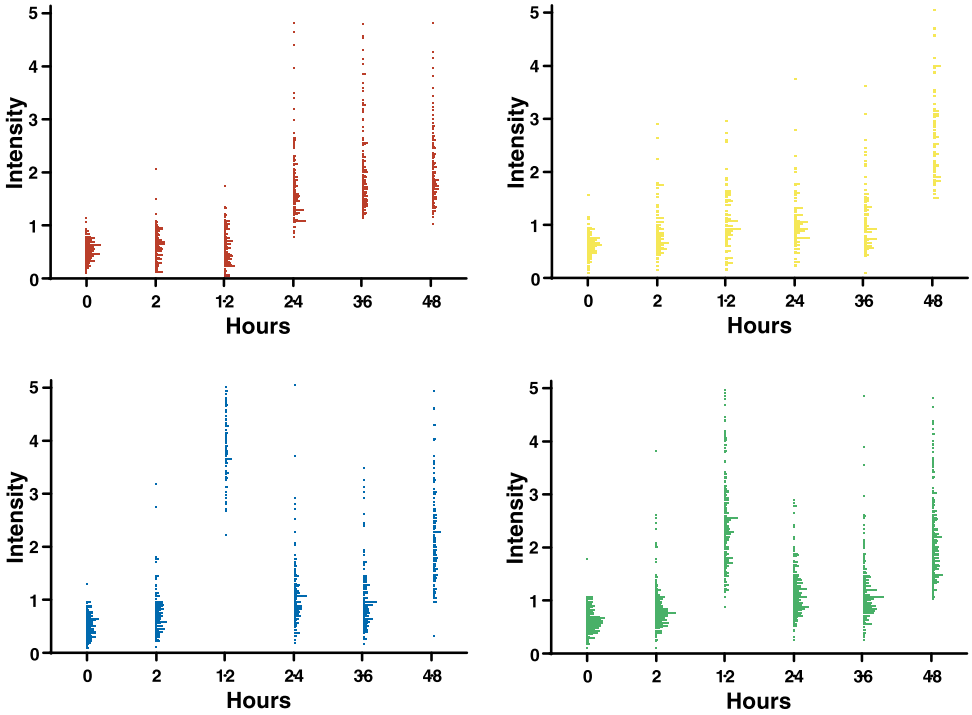


Fig. 3. K-means clustering analysis of upregulated genes for genes demonstrating a twofold or greater change in gene expression at half or more of the time points. Data shown are expression ratios on a logarithmic scale (after data normalization) where K-means clustering has placed the genes expressed at 48 h in both patient samples into 4 sets of expression patterns. Similarity in expression pattern is measured by standard correlation. For genes with no data for one-half or more of the time points, the trend is not listed. Patterns of gene expression described in text from *top left* to *top right* (*patterns 1* and *2*) and *bottom left* to *bottom right* (*patterns 3* and *4*).

a characteristic pattern of expression of a rapid increase in expression between 12 and 24 h, with a plateau of a high level of expression maintained between 24 and 48 h. Genes in this category include preprosomatostatin, somatostatin precursor I, somatostatin receptor-2 isoform (sstr2), and gene expressed in poorly metastatic human melanoma cell lines and human cerebral cortex cDNA. The coordinated pattern of regulation of three of the neuropeptide genes signaling through the STAT protein kinase cascade which follow this pattern of induction to peak levels of gene expression between 12 and 24 h and retention of high level of expression between 24 and 48 h is demonstrated graphically in Fig. 7. This well-conserved pattern of upregulation of the STAT protein kinase cascade family members implicates a coordinated regulation of these genes by cAMP.

Members of the cell death gene family follow *pattern 2* and include RANK, TRAIL, and KIAA0343 and FADD-like death effector domain. Immune genes follow two general patterns of

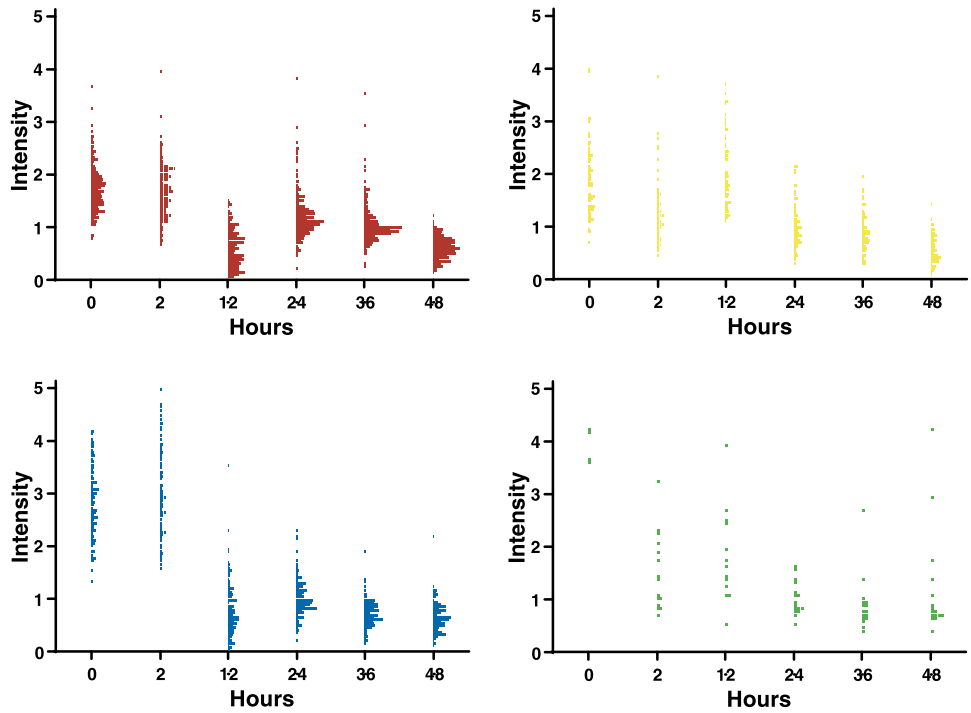


Fig. 4. K-means clustering analysis of downregulated genes for genes demonstrating a twofold or greater change in gene expression at half or more of the time points. Data shown are expression ratios on a logarithmic scale (after data normalization) where K-means clustering has placed the genes expressed at 48 h in both patient samples into 4 sets of expression patterns. Similarity in expression pattern is measured by standard correlation. For genes with no data for one-half or more of the time points, the trend is not listed. Patterns of genes expression described in text from *top left* to *top right* (*patterns 1* and *2*) and *bottom left* to *bottom right* (*patterns 3* and *4*).

Table 3. *K-means clustering analysis by ontologic (gene function) category for upregulated genes demonstrating ≥2-fold regulation of gene expression in half or more of the time points studied*

Gene Ontology Category	*Pattern 1* (induction between 12 and 24 h)	*Pattern 2* (biphasic increase in expression)	*Pattern 3* (peak in expression at 12 h)	*Pattern 4* (gradual increase in expression)
Cell growth	14	14	10	20
Cancer/cell cycle regulation	0	13	9	6
Cell death	0	15	0	0
Immune proteins	6	13	0	0
Enzymes	31	45	30	0
Nucleic acid binding proteins	0	18	5	10
Signal transduction	18	13	2	12
Structural proteins	4	4	4	4
STAT protein kinase cascade	8	5	0	0
Neuropeptides	3	0	0	0
NF-κ B protein kinase cascade	0	2	0	1
Integrin receptor signaling	0	4	4	0
GABA receptor signaling	0	3	0	0
Blood coagulation	0	2	0	3

Patterns 1–4 indicate the K-means group as depicted graphically in Fig. 3.

temporal expression. Immunoglobulin supergene family member, Ig VH5 pseudogene, and NK receptor follow *pattern 2*, and factor XIII precursor and C9 complement protein follow *pattern 1*.

Kinases displaying a pattern of negligible expression between 0 and 12 h and peaking between 12 and 24 h include Rac 1 (calmodulin binding protein), ligand for Tie2/Tek receptor tyrosine kinase and MAP kinase phosphatase. Another group of enzymes demonstrates a sharp increase in expression between 2 and 12 h, when peak levels of expression were achieved, including cytochrome *P*-450 nifedipine oxidase, carbonic anhydrase-like domain, and PPEF-2.

The majority of the nucleic acid binding proteins demonstrate a biphasic pattern of expression. The immediate early response gene NOT and its mouse homolog, NGFI-B/nur77 B type transcription factor, demonstrates biphasic regulation with marked increases in gene expression at 2 and 48 h. RNA helicase, SALL1, and Zinc finger protein ZN 72D expression increased in the first 12 h of cAMP treatment and subsequently leveled off.

The ontological subfamily of genes signaling through the NF-κB pathway achieves peak levels of gene expression at various different time points. RANK and anti-colorectal carcinoma heavy chain achieve their peak levels of expression at 2 h and 48 h, whereas TRIP9 demonstrates a gradual pattern of gene induction.

With regard to genes signaling through the integrin receptor, LFA-1 α-subunit precursor and disintegrin show a pattern of expression with peak levels achieved at 12 h and subsequently tapering off for the remainder of the time course. Members of the GABA R signaling family, including GABA transport protein, GABA-A receptor π-subunit and GABA-B receptor subunit gb2, demonstrate a biphasic pattern of expression.

Ontological Trends of Downregulated Genes

The cell growth genes exhibit two primary patterns of expression: *pattern 2* genes include IGFBP-5, monocarboxylate transporter, p55CDC mRNA, caveolae associated protein, and Na-K-ATPase β1-subunit mRNA. Genes with *pattern 3* include human nerve growth factor (HBNF-1), monocarboxylate transporter, and human histone stem-loop binding protein (SLBP). Members of the cancer/cell cycle regulation gene family exhibit two predominant patterns of downregulation seen in the K-means analysis. *Pattern 2* genes include UbcH10, proto-oncogene protein, and cyclin B2, and genes exhibiting *pattern 3* include cyclin B, associated with cyclin-dependent kinase and PRAD1 mRNA for cyclin.

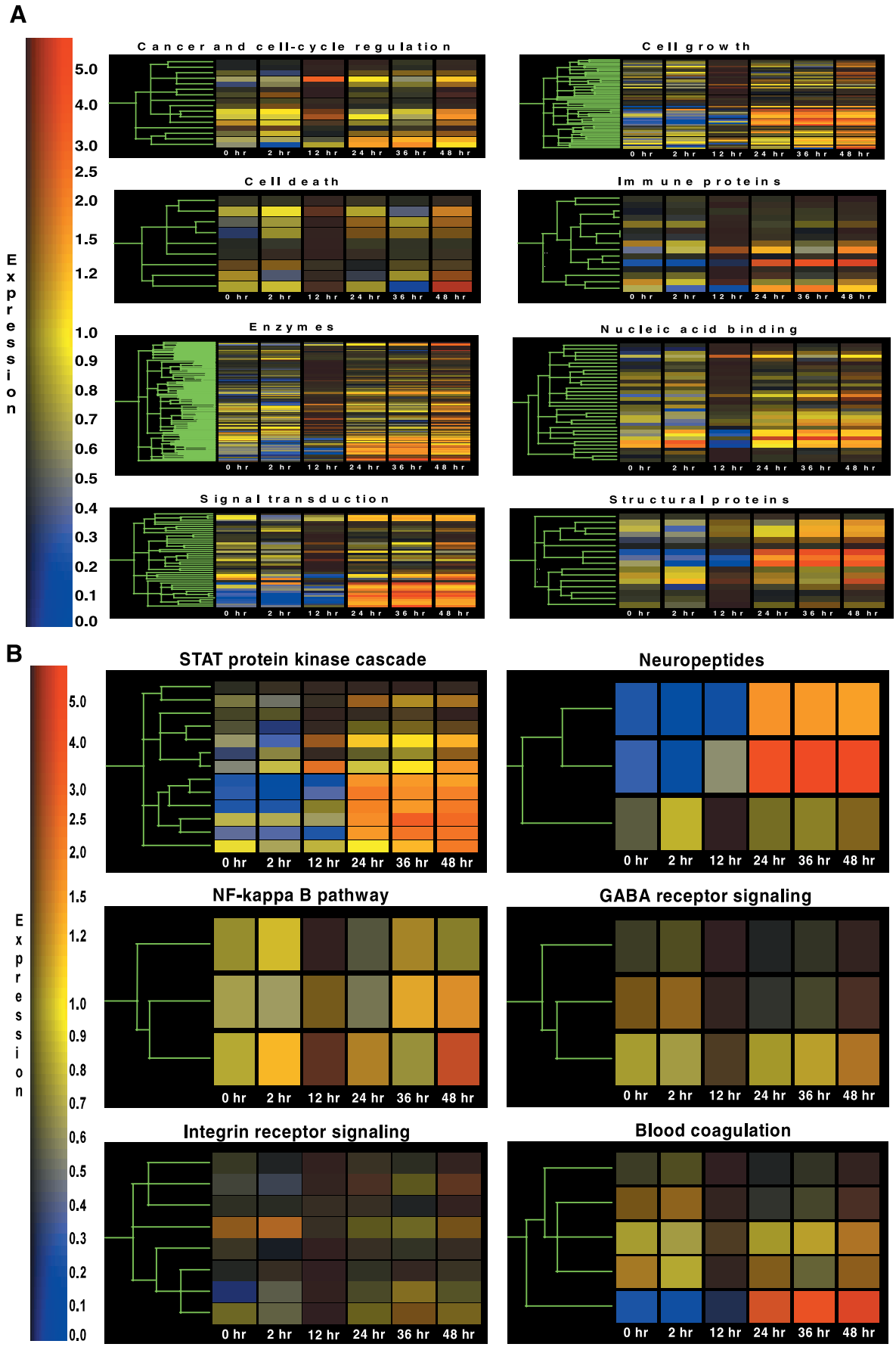
Most members of the cell death family of genes primarily exhibit the trend of *pattern 4*, including TNF ligand family, TNF type 2 receptor binding protein, and FLICE2; ICE like protease; caspase-10/b.

Kinases were found in the K-means analysis to exhibit all four predominant patterns of gene expression seen in the downregulated genes. Kinases that illustrate *pattern 1* include L-kynurenine hydrolase, argininosuccinate lyase, platelet-type phosphofructokinase, arginine-tyrosine kinase, and retSDR1. Kinase genes demonstrating *pattern 2* include thymidylate synthase, thymidine kinase, trypsinogen IV b-form, mitotic kinesin like protein-1, *N*-myristoyltransferase-2, nicotinic mononucleotide pyrophosphorylase, and Na-K-ATPase β1-subunit. Genes demonstrating *pattern 3* include phospho*enol*-pyruvate carboxykinase, P-13 kinase associated p85 mRNA, retinal short-chain dehydrogenase/reductase, and pre-β-migrating plasminogen activator inhibitor. Kinases demonstrating *pattern 4* include aldolase A, glyceraldehyde-3-phosphate dehydrogenase, disintegrin and metalloprotease-like domains, and nicotinate mononucleotide pyrophosphorylase.

Table 4. *K-means clustering analysis by ontologic (gene function) category for downregulated genes demonstrating ≥2-fold regulation of gene expression in half or more of the time points studied*

Gene Ontology Category	*Pattern 1* (downfold induction throughout time course)	*Pattern 2* (upregulation 0–2 h; persistent downregulation 12–48 h)	*Pattern 3* (biphasic decrease in expression at 12 and 48 h)	*Pattern 4* (upregulation to 12 h and downregulation from 24–48 h)
Cell growth	0	8	16	0
Cancer/cell cycle regulation	5	7	3	4
Cell death	0	1	1	4
Immune proteins	0	3	1	4
Enzymes	20	29	31	11
Nucleic acid binding proteins	0	10	1	3
Signal transduction	9	21	0	15
Structural proteins	0	3	0	0
G protein receptor signaling	2	3	0	0
Extracellular matrix	1	5	0	0
Serine-threonine kinase	2	3	0	0
Integrin receptor signaling	1	3	0	1

Patterns 1–4 indicate the K-means group as depicted graphically in Fig. 5.



The nucleic acid binding protein family of genes exhibits primarily *pattern 1* and includes winged helix transcription factor, DNA binding protein AP-2, ERCC2 gene, hnRNAcore protein A1, and myocyte-specific enhancer factor 2A (MEF2A).

The immune gene family demonstrates three predominant patterns of expression, *patterns 1*, *2*, and *4*. Genes following *pattern 1* of expression include placental protein 14 (glycodelin); genes following *pattern 2* of expression include Fc-γ-RIIA gene for IgG Fc receptor and MHC class I mRNA fragment. Genes following *pattern 4* of expression include skeletal muscle 165-kDa protein and CMRF 35 mRNA.

Genes for receptors or genes playing a direct role in signal transduction followed three major patterns of expression according to the K-means analysis. *Pattern 1* was exhibited by activator of NF-κB, integrin-α4 subunit, arginine-tyrosine kinase, soares pregnant uterus [decidual/trophoblast PRL-related protein (d/tPRP)], and FRP (frizzled homolog). Another set of genes follows *pattern 2*, including GnRH receptor, keratin 18 precursor, γ-interferon-inducible protein precursor, RTP gene, pregnancy-specific β-1 glycoprotein precursor, integrin-α6, and serine/threonine kinase (BTAK). The other set of genes in the signal transduction family follows *pattern 4* and includes testis-specific disintegrin and metalloprotease-like domains, and annexin homologous tetrad.

Genes of the structural protein family exhibit predominantly *pattern 2* and include actin-, tropomyosin-, and calmodulin-binding protein in smooth muscle, α1 type XVI collagen (COL16A1), and preprofactor XI. Genes signaling through G protein receptors follow either *pattern 1*, including G-protein-coupled receptor, purinergic P2YU receptor and BLR1 gene for Burkitt's lymphoma receptor, or genes following *pattern 2*, including endothelial differentiation protein (edg-1), type 3 inositol 1,4,5-triphosphate receptor, cytosolic thyroid hormone-binding protein, and γ-interferon-inducible protein precursor. Genes in the extracellular matrix family of genes exhibit predominantly *pattern 2* and include α1 collagen type XIII mRNA, keratin 18 precursor, and α1 type XVI collagen (COL16A1). Genes in the integrin receptor signaling pathway exhibit primarily *pattern 2*, including integrin-α6 and integrin-α4 subunits. Other genes in the integrin family exhibit *pattern 1* and include eMDC II protein. Genes in the serine/threonine kinase family exhibit either *pattern 1* or *pattern 2*. Genes following *pattern 1* include cdk3 for serine/threonine protein kinase. Serine/threonine kinase family members following *pattern 2* include serine/threonine kinase (BTAK) and murine pim-2 product.

Validation of Gene Expression

Northern analysis and RT-PCR with RNA from endometrial stromal cells treated with and without cAMP were conducted to validate select gene expression. RT-PCR (see results in Fig. 8) was performed with cells treated with 1 mM cAMP for 48 h, where the levels of gene expression in these cells were compared with those of nontreated stromal cell RNA (*time 0*). The primer sets utilized for RT-PCR are shown in Table 1. Although quantitative RT-PCR was not performed, it is evident from the data shown in Fig. 8, *A* and *B*, that there is clear upregulation in the human endometrial stromal cell of preprosomatostatin, IGFBP-1, and IL-11 and downregulation of IGFBP-5, TIL-4, and cyclin B with treatment with cAMP. These data are consistent with observations from the microarray data and Northern analysis (see below). In addition, downregulation of IGFBP-5, cyclin B, and TIL-4 was validated by RT-PCR in Fig. 8, *A* and *B*.

Northern analysis was performed with RNA from two different patient samples, utilizing a time course of treatment with cAMP similar to that of the microarray experiment, to observe the sequential pattern of gene expression (Figs. 9, *A–G*). Selected genes studied include IL-11, preprosomatostatin, and IGFBP-1, where the pattern of expression was studied at 0, 6, 12, 24, and 48 h. Some of the genes utilized in our validation, IL-11, IGFBP-1, preprosomatostatin, cyclin B, and IGFBP-5, have been previously shown to be regulated with decidualization. These were selected for their abundant level of expression, which allowed their detection (as early as 6 h in the time course) by Northern blot analysis. Furthermore, genes with a high level of expression throughout the time course were also selected for validation so that a comparison of the kinetics of microarray-derived gene expression could be compared with the results from the Northern blots. This is shown in Fig. 10, *A* and *B*, for preprosomatostatin and IL-11, respectively. The significant downregulation of TIL-4 with cAMP treatment was a novel finding in our array, and thus validation of this genes' expression pattern utilizing RT-PCR confirmed this novel finding in our microarray data. In addition, validation of other genes in response to cAMP which are known to be expressed in decidualized endometrium, i.e., IL-11, IGFBP-1, preprosomatostatin, IGFBP-5, and cyclin B, further validated the in vitro cell model system.

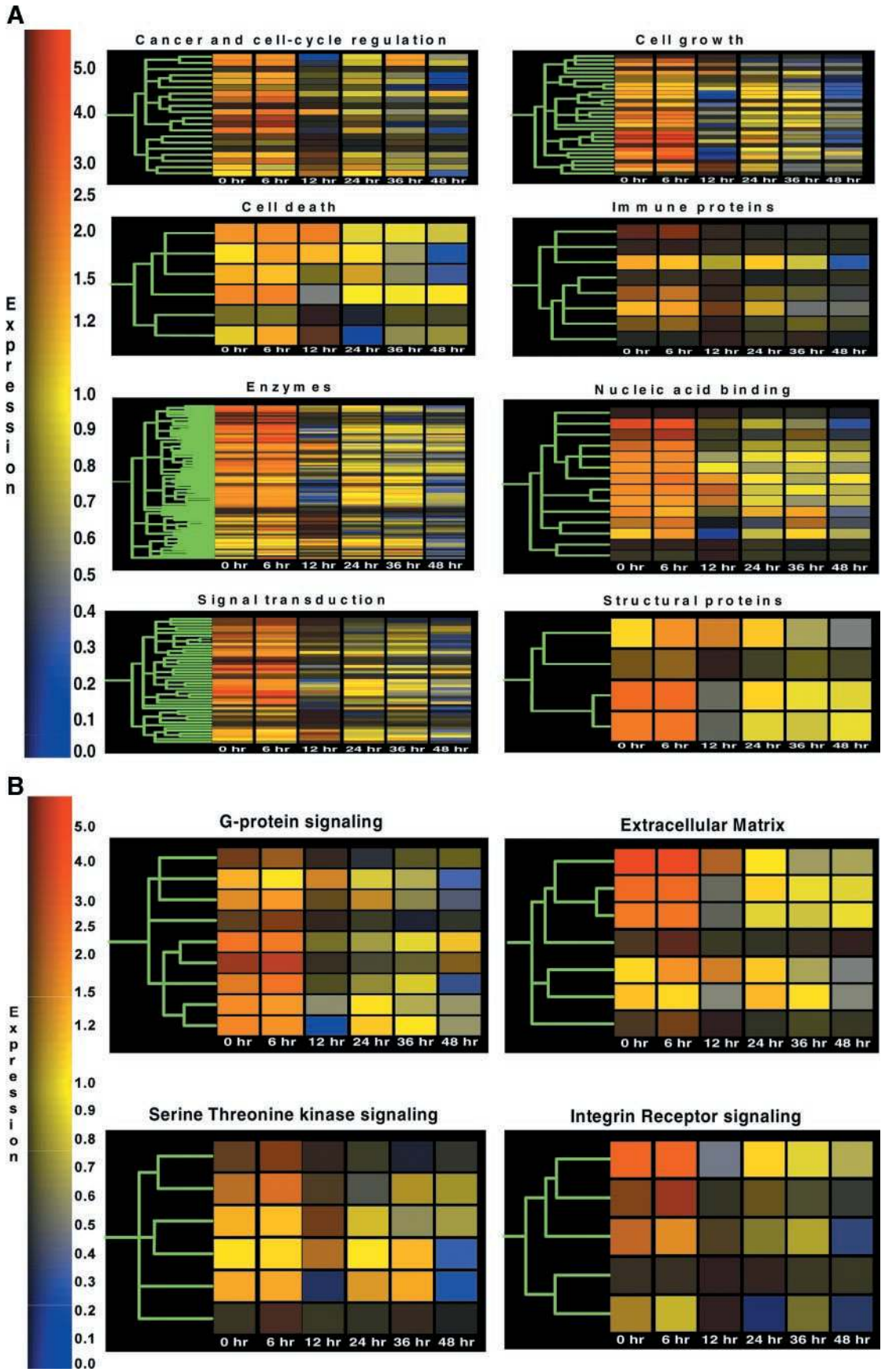
Densitometric analysis was conducted to compare fold changes between mRNAs in Northern analysis, after normalization to GAPDH. Comparison of the trends in gene expression determined by Northern analysis and by microarray analysis is demonstrated in Fig. 10, *A* and *B*. Northern analysis was consistent with the sequential pattern of expression of preprosomatostatin, IGFBP-1, and IL-11 observed in the microarray analysis.

DISCUSSION

Gene Families Regulated by cAMP Treatment

In this study, because the endometrial stromal cell cultures are 99% pure, it can be concluded that the genes regulated by cAMP treatment reflect the specific response of the stromal cell to the activation of the PKA pathway. Some of the genes identified in this study have previously been reported to be regulated in the stromal cell upon decidualization with cAMP

Fig. 5. *A*: expression ratio (after data normalization) of upregulated genes for the eight major ontological categories (cancer and cell cycle regulation, cell growth, cell death, immune proteins, enzymes, nucleic acid binding, signal transduction, and structural proteins) at 0, 2, 12, 24, 36, and 48 h. *B*: expression ratio (after data normalization) for six ontological subcategories (STAT protein kinase cascade, neuropeptides, NF-κB pathway, GABA receptor signaling, integrin receptor signaling, and blood coagulation) at 0, 2, 12, 24, 36, and 48 h.



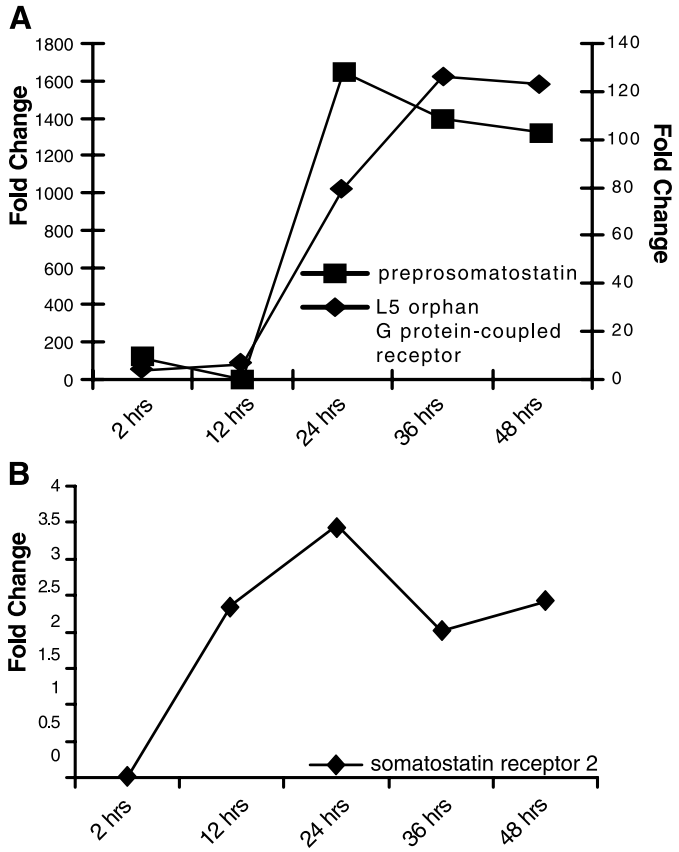


Fig. 7. Microarray expression of selected neuropeptide genes: preprosomatostatin and L5 orphan G protein-coupled receptor (*A*) and sstr2, human somatostatin receptor isoform 2 (*B*). Data shown are mean fold change at each time point relative to 0 h time point.

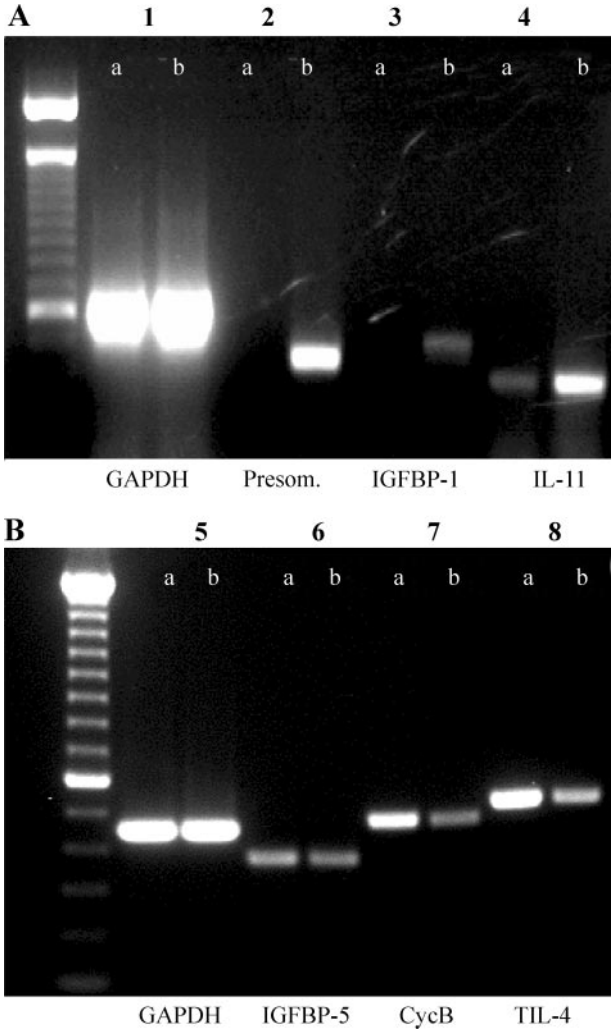


Fig. 8. Validation of selected genes which were upregulated (*A*) or downregulated (*B*) during decidualization in human endometrium by RT-PCR. Endometrial stromal cell culture samples at 0 h (prior to onset of decidualization) (*lane a*) and 48 h of decidualization (*lane b*) with 1 mM cAMP were processed for total RNA, and RT-PCR was performed with the primer sets shown in Table 1. Appropriate size products were found for GAPDH (internal control) (*lane 1*), preprosomatostatin (*lane 2*), IGFBP-1 (*lane 3*), and IL-11 (*lane 4*); GAPDH as a control (*lane 5*), IGFBP-5 (*lane 6*), cyclin B (*lane 7*), and TIL-4 (*lane 8*), respectively.

and/or E2/P4 in vitro, including, IGFBP-1, IL-11, inhibin β_B, IL-11, TGF-β1, TGF-β2, and VEGF (24). In addition, several of the genes we found, for example, sstr2, IGFBP-2, and MMP-10, are known to be upregulated in secretory endometrium (12). However, the majority of genes found in the current study have not previously been identified in decidualized endometrium or expressed during the process of decidualization. Furthermore and importantly, the kinetic patterns of gene expression, with regard to the timing of mRNA induction during the course of treatment with cAMP, have not been previously reported.

Since the two cell lines studied were matched for IGFBP-1 levels achieved at each time point (0, 2, 12, 24, 36, and 48 h) (see Fig. 2), the level of differentiation in response to cAMP was not likely to contribute significantly to the variability in the levels of gene expression. Similar levels of IGFBP-1 secreted by the two patient samples have been assumed to reflect similar degrees of differentiation. Thus, although the pattern of gene expression was consistent throughout the time course, the magnitude of expression at individual time points during decidualization differed likely due to patient-to-patient variability.

The most significantly upregulated genes detected in this study include preprosomatostatin, IGFBP-1, NOT, MMP-10, orphan L5 G-protein-coupled receptor, prolactin, and human negative growth protein MYD 118. The most significantly downregulated genes include activator of NF-κB, actin/troponin/tropomyosin binding protein, IGFBP-5, lipocortin III, retinal short chain dehydrogenase, and α1 type XVI collagen. Several genes and gene families found herein to be sequentially regulated during treatment with cAMP over a 48-h period were similar to those recently reported with human term pregnancy decidual fibroblasts treated for 15 days with cAMP and E2/P4

Fig. 6. *A*: expression ratio (after data normalization) of downregulated genes for the eight major ontological categories (cancer and cell cycle regulation, cell growth, cell death, immune proteins, enzymes, nucleic acid binding, signal transduction, and structural proteins) at 0, 2, 12, 24, 36, and 48 h. *B*: expression ratio (after data normalization) for four ontological subcategories (G protein signaling, extracellular matrix, serine threonine kinase signaling, and integrin receptor signaling) at 0, 2, 12, 24, 36, and 48 h.

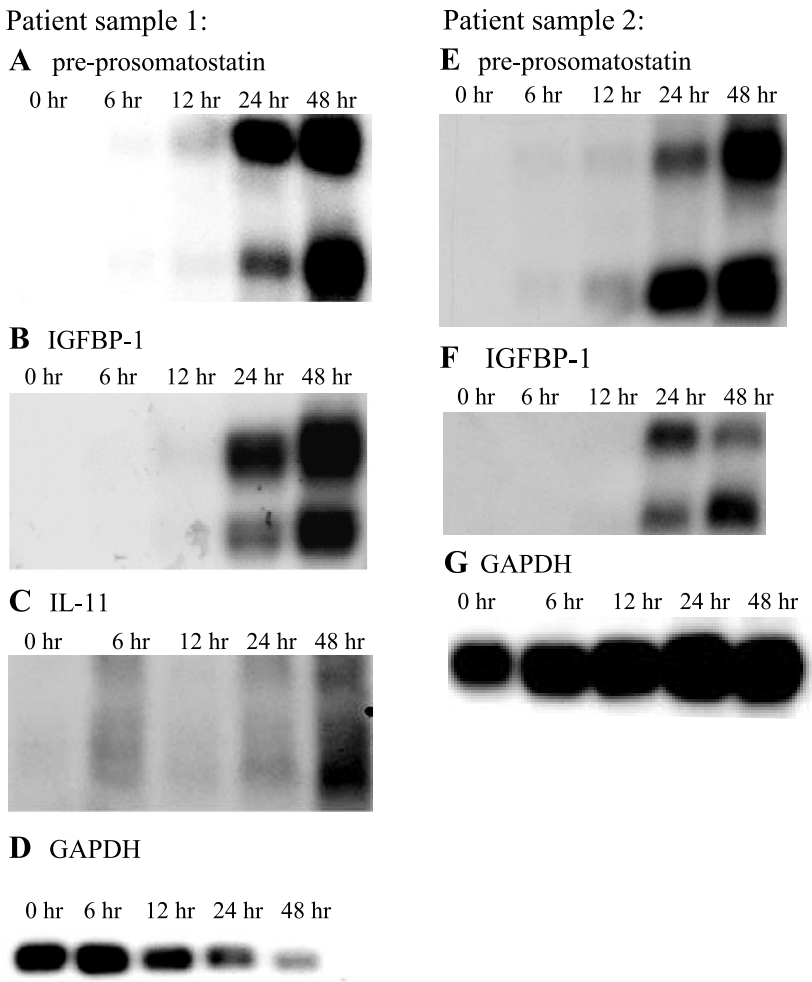


Fig. 9. Northern analysis demonstrating sequential upregulation in the two patient samples of the appropriate size gene products for IGFBP-1 (*B* and *F*), preprosomatostatin (*A* and *E*), and IL-11 (*C*) with decidualization in the human endometrial stromal cell with 1 mM cAMP. *D* and *G*: GAPDH hybridizations of respective blots are shown for comparison.

(6). The comparison of the results of the study of Brar et al. (6) and our current microarray analysis for some of the most significantly upregulated and downregulated genes is displayed in Table 5. The consistency between these data with different sources of stromal cells (pregnancy decidua vs. cycling, nonpregnant endometrium), time courses, and differing decidualization stimuli, remarkably underscores the validity of the data and the commonality of some genes expressed during development of the decidual phenotype.

Many of the genes identified in our current study were also found in a previous microarray study in our laboratory by Popovici et al. (39), in which a comparison was performed of the gene expression profile of cells treated with cAMP for 48 h and of cells treated with E2/P4 for 10 days. In this study (39), Atlas cDNA arrays were utilized, which contained a significantly smaller subset of genes and expressed sequence tags (ESTs) (~500) relative to the Affymetrix Hu95A oligonucleotide GeneChip utilized in the current study (over 12,000 genes and ESTs). Table 6 demonstrates a comparison between the results of Popovici et al. (39) and the results of our current study, both of which analyzed changes in gene expression upon treatment with cAMP for 48 h.

The increased fold upregulation of many of these genes in our current study relative to our previous microarray study (39) may be attributed to the increased responsiveness to cAMP and the increased levels of IGFBP-1 secretion (1,500–2,000 ng/ml at 48 h), representing an overall increased level of differentiation to the decidualized phenotype in this experiment relative to our previous microarray study (39).

K-means Analysis

K-means analysis revealed four predominant patterns of gene expression for the genes for which there was greater than twofold regulation in our time course microarray. Ontological categories of genes contained similar trends of gene regulation, indicating that there is coordinated regulation of genes by function that mediate the secretory and morphologic transformation of the human endometrial stromal cell during decidualization.

Upregulated Gene Families

Neuropeptides. Remarkable changes in levels of expression were observed for the somatostatin receptor signaling pathway, with coordinated induction of preprosomatostatin, somatostatin R isoform 2 and the orphan G-protein-coupled receptor. Immunohistochemical studies have localized somatostatin to secretory endometrium, exclusively in the endometrial stromal

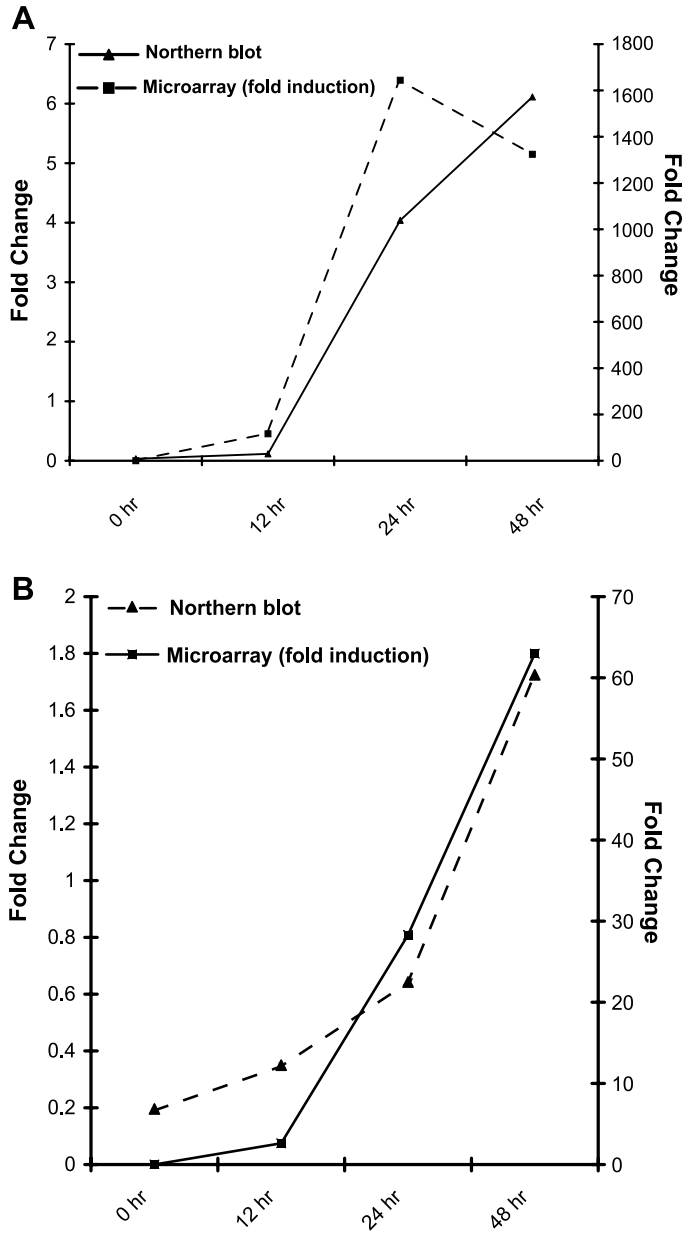


Fig. 10. Comparison of mean values for Northern blot densitometry (normalized to GAPDH) for the two patients studied and microarray fold induction (each time point normalized to the gene expression at 0 h) at 0, 12, 24, and 48 h for two of the genes studied: preprosomatostatin (*A*) and IL-11 (*B*). Note: One patient sample was utilized for both microarray and Northern blot analysis, and the two other samples were utilized for either Northern blot or microarray analysis. All samples utilized were matched for peak IGFBP-1 levels of 1,500–2,000 ng/ml at 48 h.

cells (27), and upregulation with decidualization is consistent with a recent study (6). The somatostatin receptor sstr2 is expressed in endometrial epithelium, endothelium, and stroma throughout the menstrual cycle. Its expression varies in the epithelial cells surrounding the endometrial glands from being basal or diffuse in the proliferative and secretory phase, respectively, to being lumenal in the menstrual stage (19). Somatostatin is best known for its function in inhibiting pituitary GH release upon binding to sstr2 (44). However, it also inhibits the release of other physiologically important compounds, including insulin, glucagon, gastrin, and secretin (46). Somatostatin also functions as a promoter and an inhibitor of angiogenesis, depending upon the tissue type studied (32, 35). For example, with the pituitary tumor cell line TtT/GF the somatostatin analog, octreotide, stimulates release of VEGF (32), whereas with human umbilical endothelial cells, it inhibits its basal and stimulated endothelial cell proliferation (35). Somatostatin further exhibits both inhibitory and stimulatory effects on immune cell proliferation and secretion of cytokines (22, 29), including release of IL-6 from peripheral blood monocytes (29). Somatostatin binds to target cells via a member of the seven transmembrane domain superfamily of glycoprotein receptors, leading to inhibition of adenylate cyclase and Ca^{2+} channel activity, stimulation of K^+ and tyrosine phosphatase activity, and regulation of intracellular pH (46). We postulate that somatostatin may act in an autocrine fashion via sstr2 to inhibit proliferation and promote the differentiation of the decidualized stromal cell, in addition to acting by paracrine mechanisms to regulate angiogenesis and immune functions during endometrial cyclic changes and implantation.

Semaphorin III, upregulated during stromal cell treatment with cAMP herein, was shown in a recent report from our group to be downregulated during the window of implantation in human endometrium (28). These differences may reflect semaphorin expression in other cell types in whole tissue or paracrine interactions required for cellular expression in vivo that are not preserved in isolated cell culture systems in vitro. Semaphorins are a family of neuropeptides that act as chemoattractants or repellants, depending upon levels of cGMP (29). The finding of semaphorin expression in the endometrium and its regulation with decidualization have led us to hypothesize that chemoattractants and ion signaling may function to guide an embryo in the endometrium during implantation (28).

Table 5. *Comparison of present results with those of Brar et al. (6)*

	Fold of Regulation with 15 days of 8-Br-cAMP and E2/P4, Brar et al. (6)	Fold of Regulation with 48 h of 8-Br-cAMP (Current Microarray Study)
Upregulated genes		
IGFBP-1	131.4	115.8
EBAF	44.4	51.4
Somatostatin	19.8	1342
Kip 2	3.6	7.8
Downregulated genes		
IGFBP-5	20	11.2
α1 Type XVI collagen	3.3	44.6

Table 6. *Comparison of present results with those of results of Popovici et al. (39)*

Gene Name	Fold Upregulation with 48 h of 8-Br-cAMP, Popovici et al. (39)	Fold Upregulation with 48 h of 8-Br-cAMP (current microarray study data)
IL-11	2.8	62.9
IL-8	5.2	19.7
TGF-β2	5.5	11.1
Activin β_B-subunit	7.9	55.5
TNFα	1.3	2.3
TRAIL ligand	2.9	3.95

Immune genes. Some of the immune genes of interest found to be sequentially upregulated with cAMP treatment are demonstrated in Table 7. The response of IL-8, IL-11, and the IL-1 receptor is consistent with work previously published (39). IL-11 upregulation with cAMP treatment is of particular interest, as IL-11 mRNA expression in endometrial stromal cells during the secretory phase precedes that of prolactin, a known marker of decidualization (10). Furthermore, in mice, a null mutation in the IL-11 Ra subunit results in an infertility phenotype, because of the absence of stromal decidualization (1). In addition, IL-11 enhances stromal cell viability and prolactin secretion from cells decidualized with 8-Br-cAMP (52). Thus IL-11 has an important role during the process of decidualization and stromal cell survival.

TNF-α, upregulated with cAMP treatment, has been shown by immunoassay to be secreted from stromal cell cultures in vitro (3) and may stimulate stromal cell proliferation by induction of IL-8 (26). Receptor activator of nuclear factor and κB (RANK), involved in the function and development of osteoclasts (36) and expressed in osteolytic bone tumors (36), has not previously been shown to be expressed in the endometrium and intriguingly may serve as a signaling receptor during decidualization. TNF-related apoptosis inducing ligand (TRAIL), upregulated in our previous cDNA microarray study (39) and herein, is a killer of activated lymphocytes, suggesting a role for the decidua in protecting an implanting cytotrophoblast from destruction by the maternal immune system (39).

Cell growth and cell differentiation. A variety of genes involved in promoting and regulating cell growth and differentiation were found to be upregulated herein, including VEGF, TGF-β family members, and specific early response genes. VEGF is expressed by the stromal cells during the midsecretory phase, and both VEGF immunostaining intensity and mRNA expression are significantly increased by the administration of E2/P4 to stromal cells in vitro (2), consistent with the results of our previous cDNA microarray study (39). VEGF is postulated to play an important role in endometrial angiogenesis and implantation and in the maintenance of pregnancy. Herein, we found upregulation of EBAF, another TGF-β family member. In fertile women, EBAF expression decreases during the window of implantation and increases in late secretory and menstrual endometrium (50). In contrast, in women with infertility and endometriosis, EBAF expression does not decrease during the window of implantation, and it has been hypothesized that it may be a marker for uterine nonreceptivity (50). Other interesting genes involved in cell growth that are upregulated with cAMP treatment include FGF-18 and FGFR2, PDGF-αR, hepatocyte growth factor, β nerve growth factor, and connective tissue growth factor related protein WISP-1 subunit precursor, and TGF-β2 precursor, were upregulated the findings herein.

Prolactin was found to be significantly upregulated with cAMP treatment in our array (143.4-fold above *time 0* at 36 h, 37.135-fold above *time 0* at 48 h), which is consistent with previous studies which have found prolactin to be one of the major secreted proteins of the decidualized endometrial stromal cell (55).

Some immediate-early response genes with significant homology to steroid and thyroid hormone receptors were regulated during stromal cell treatment with cAMP. The NOT gene exhibited marked upregulation after 2 h of treatment with cAMP (239.4 and 462.5-fold above *time 0*), and NGFI-B/nur77, the mouse and rat homolog of the human immediate-early response gene NAK1/TK3, was expressed at 47.8 and 164.5-fold at 2 h, with a subsequent decease for the duration of our time course. NOT has previously only been detected in vivo in the brain (33). NOT and NAK1/TK3 represent a distinct group of orphan steroid receptors that function as general coactivators of gene transcription, rather than as typical steroid receptors that act to induce specific target genes (33). In vitro NOT mRNA is expressed in growth-arrested fibroblasts within 30 min of treatment with serum, and peak levels are achieved by 3 h (33). This response is not affected by cycloheximide, suggesting that NOT expression is consistent with an immediate-early response gene and does not depend on protein synthesis for expression (33). The impressive upregulation of both NOT and NAK1/TK3 within a short time frame in endometrial stromal cells in response to cAMP treatment suggests early transcriptional activation that may be important in the transition to the decidual phenotype from proliferation to production of unique extracellular matrix and secretory products (see below).

Oncogenes/cell cycle regulators. Several genes involved in cell cycle regulation are regulated with cAMP treatment, including snoI, N-ras, NF1, Rb-related protein (p107), trk, and Kip 2; snoI is an oncogene with a proposed role in muscle gene regulation (38), and N-ras is an oncogene found in a subset of endometrial carcinomas (56). NF1 (neurofibromin 1 gene) is implicated in the pathogenesis of neurofibromatosis type 1 (30) and was also found to be upregulated in our previous cDNA microarray study (39) and in decidualization of term decidual fibroblasts (53). Its function in the endometrium is enigmatic. Retinoblastoma-related protein (p107), a known tumor suppressor gene, may have a role during development and differentiation (8). Abundantly expressed in placenta during the first trimester, it has been postulated to control trophoblast proliferation (8), and it may play a role in differentiation of the endometrial stromal cell. Trk is a high-affinity neurotrophin

Table 7. *Summary of immune gene upregulation*

Immune Gene	Fold Upregulation at 48 h (relative to 0 h)
IL-11 (adipogenesis inhibitory factor)	63
IgG Fc fragment R precursor	27.21
Factor XIII precursor	25.8
FHR-2 (complement related factor H protein)	14.5
IL-8	13.1
C9 complement protein	7.5
Monocyte/macrophage Ig-related putative inhibitory receptor	6.3
Ig superfamily gene member	6.24
CD20 (B-lymphocyte cell surface antigen B1)	5.6
Semaphorin III	3.9
TNF-related apoptosis inducing ligand (TRAIL)	3.9
Killer cell immunoglobulin-like receptor	3.7
Ig VH4 heavy chain	3.4
IL-2	3.3
IL-1 type I receptor	3
TNF-α inducible protein	2.9
Receptor activator of nuclear factor and kappa B (RANK)	2.6
TNF-α	2.6

Values indicate fold upregulation at 48 h relative to gene expression at 0 h.

receptor, and TrkA and TrkC have been previously shown to be specifically expressed in secretory-phase endometrium (45). The presence of neurotransmitters, neurotropic factors, and their receptors in the endometrium requires further investigation. The CDK inhibitor Kip 2 is a gene implicated in tumorigenesis; however, there is no previous evidence of expression or tumor induction in the endometrium by Kip 2 (42). The BRCA genes are linked to a variety of reproductive tract tumors, including uterine and ovarian serous papillary carcinoma and carcinoma of the breast (23). The finding of upregulation of cell cycle regulatory genes in the decidualized stromal cell lends support to the observed cytological evidence that during the decidualization transition, endometrial stromal cells expand not only by cellular hypertrophy, but also by mitosis and endoreduplication. Proliferation of endometrial stromal cells is mediated by growth-related peptides, prostaglandins, and Ki67 (15). Roles for these cell cycle regulators in endometrial stromal cellular decidualization await further study.

Extracellular matrix. The extracellular matrix undergoes marked transformation in the establishment of the decidua and in nonpregnancy cycles in preparation for menstrual tissue desquamation (41). Herein, MMP-10 (stromelysin-2) is markedly upregulated during cAMP treatment. Previous studies have shown stromelysin-2 mRNA is expressed in stromal cells in late secretory and menstrual endometrium (41), consistent with the current study. The pattern and temporal expression of MMP-10 suggest that it may play a key role in the decidualized stromal cell for matrix remodeling. Other genes found to be sequentially upregulated during the cAMP treatment time course include collagenase 3 and extracellular matrix protein.

Cholesterol trafficking and transport. Apolipoprotein E (ApoE) has previously been shown to be highly upregulated in endometrial tissue during the window of implantation (28) and herein, receptor 2 for ApoE was upregulated during decidualization. ApoE, produced locally in steroidogenic tissues, e.g., the ovary, binds to hydrophobic molecules and functions in cholesterol transport and trafficking (34). Upregulation of ApoE receptor 2, apolipoprotein A1, and apolipoprotein CII with cAMP treatment suggests that these molecules may play an important role in cholesterol transport or steroid hormone activation in the endometrium.

NF-κB protein kinase cascade. One member of the NF-κB protein kinase cascade that is upregulated with cAMP treatment is the thyroid receptor interactor (TRIP9). TRIPs are ligands to the thyroid hormone receptor and are dependent upon thyroid hormone for interaction with the receptor (31). TRIPs also show similar ligand-dependent interaction with the retinoid X receptor (RXR), although they do not interact with the glucocorticoid receptor. Another member of this signaling family that is upregulated during stromal cAMP treatment is glycoprotein CANAG-50-specific IgG1-κ. The NF-κB pathway is known to play a role in the baboon endometrium during decidualization (48). Current data suggest that IL-1β activates multiple signaling pathways that either positively (in the absence of exogenous cAMP) or negatively (in presence of exogenous cAMP) regulate decidualization and IGFBP-1 gene expression in vitro, involving NF-κB activation as well as phosphorylation of p38 MAPK (48).

GABA receptor signaling. The GABA-A receptor π-subunit, upregulated with cAMP treatment, is abundantly and specifically expressed in the rat uterus (13) and was found to be upregulated in our previous microarray studies during stromal decidualization (39) and in the implantation window of human endometrium (28). The π-subunit decreases responsiveness to progesterone metabolites, such as allopregnanolone, and may inhibit uterine contractility prior to the onset of labor (13). The presence of this subunit in the decidualized stromal cell suggests a role for it during implantation, perhaps by modulating the effects of ligands such as GABA and progesterone metabolites to the GABA-A π-receptor.

Integrin receptor signaling. LFA-1 (leukocyte function-associated antigen-1) α-subunit precursor is associated with integrin receptor signaling. LFA-1 has been shown to increase in secretory endometrium, suggesting that its expression is hormonally dependent (14), consistent with the current study. LFA-1 plays a critical role in promoting NK cytolysis in peripheral blood lymphocytes, through potential mechanisms such as promoting cell-mediated target cell adhesion, lysis and apoptosis (14). It may be important in early pregnancy, as evidenced by significantly increased LFA-1 levels in decidual CD56 NK cells in women with spontaneous abortion, relative to normal pregnancy (14). Other genes upregulated with cAMP treatment, herein, include MDC2α/MKC2β, disintegrin, and B-6 extracellular matrix receptor.

Cell adhesion. Several cell adhesion genes were upregulated during cAMP treatment. For example, sialophorin, CD43, is a sialoglycoprotein expressed on the surface of a wide variety of blood cells including T lymphocytes. Its ligation induces proliferation and activation of human T lymphocytes (4). Intraepithelial leukocytes expressing CD43 increase from the proliferative to the late secretory phase, where higher levels of CD43-positive cells are found in the surface epithelium compared with glandular epithelium, and it is also expressed in stroma (7). It is hypothesized that during the secretory phase of the menstrual cycle and in early pregnancy, specific leukocyte recruitment to the endometrium limits the type of immune cells which gain access to the endometrium (4). One of these leukocyte recruitment molecules, neural cell adhesion molecule (NCAM), is upregulated with cAMP treatment. Previous studies have also shown that decidual infiltrating lymphocytes express NCAM, and thus it is hypothesized to play a role in endometrial lymphocyte recruitment and adhesion (4).

Cell death. Several genes with functions in cell death were found to be upregulated with endometrial stromal cell cAMP treatment including T cell death associated protein, Bik, inhibitor of bcl-2, and BH3 interacting domain death agonist BID. BIK, a pro-apoptotic member of the bcl-2 family, is induced by p53 stimulation of apoptosis (16). BIK may act to induce cell-death by its known role in the initiation of cytochrome *c* release from the mitochondria and in the induction of caspase expression (16). It is unclear why cell death genes are induced in stromal cells treated with cAMP, since the majority of apoptosis during the menstrual cycle occurs in the epithelium (11).

Downregulated Genes

Some of the most markedly downregulated genes upon cAMP treatment include activator of NF-κB, L-kynurenine hydrolase, actin/tropomyosin/calmodulin binding protein, cyclin B, IGFBP-5, lipocortin III, FRP (frizzled homolog), and cyclin E2. The activator of NF-κB (average downfold regula-

tion of 369 at 48 h, relative to *time 0*), is a member of the cytokine-mediated IL-1R/I-κB/NF-κB activation cascade (9). It exhibits structural and functional similarities with the *Drosophila* Toll/Cactus/Dorsal signaling pathway, where the homologous *Drosophila* gene Toll (dToll) regulates dorsal-ventral polarity in the developing embryo and activates the innate immune response in the adult fly (9). Recent evidence suggests that a human homolog of the dToll protein, TIL-4 (found to be significantly downregulated, Supplemental Table A), participates in the regulation of both innate and adaptive human immunity through the activation of NF-κB and the expression of the NF-κB-controlled genes IL-1, IL-6, and IL-8 (9). The human TIL-4 (Toll/IL-1R-like-4) gene exhibits homology to both the leucine-rich repeat extracellular domains and the IL-1R-like intracellular domains of *Drosophila* Toll (9). Functional studies showed that TIL-4 activates NF-κB in a cell type-dependent fashion (9). As the TIL-4 gene is believed to play a similar role in immune regulation as its *Drosophila* homolog Toll, downregulation of TIL-4 in the decidualized stromal cell may play a key role in inhibiting the maternal immune response to the invading cytotrophoblast and the embryo during implantation, as well as in regulation of endometrial stromal cell decidualization.

IGFBP-5 mRNA is the only member of the IGFBP family with increased expression in the proliferative phase vs. the secretory phase of the menstrual cycle, consistent with our finding of downregulation of IGFBP-5 upon cAMP treatment of the stromal cell (59). IGFBP-5 demonstrates a diffuse stromal pattern of expression in the endometrium (59). The IGF system plays a fundamental role in endometrial biology, acting via autocrine and/or paracrine mechanisms, with IGF-I and IGFBP-5 being dominant in the proliferative phase, and IGF-II and the other IGFBPs predominant in the secretory phase of the menstrual cycle (59).

L-Kynurenine hydrolase, an enzyme involved in the pathway of tryptophan metabolism, was downregulated by 169.3-fold at 48 h. L-Kynurenine hydrolase activity is inhibited by natural and exogenous estrogen, as evidenced by increased urinary excretion of tryptophan metabolites upon treatment with estrogen (57). Evidence suggests that this inhibition is an estrogen-mediated decrease in the availability of vitamin B_6, the coenzyme of kynureninase, although estrogen may also exhibit a direct effect on kynureninase activity (57). The roles in endometrium for this interesting gene may involve upregulation of vitamin B6 availability in this tissue during the secretory phase of the menstrual cycle.

Phospholipase C hydrolysis of phosphoinositide (PI) results in the generation of cyclic and noncyclic inositol phosphates (51). Cyclic inositol phosphohydrolase (cIPH) is a phosphodiesterase that cleaves the cyclic bond of one of the products of the phospholipase C reaction, cyclic inositol monophosphate (51). It has recently been purified from the human placenta and is identical to lipocortin III (51), a gene found to be markedly downregulated during cAMP treatment (Supplemental Table A) and during the window of implantation (28). Lipocortin III is also known as annexin III and as placental anticoagulation protein III. Regulation of PI intermediates is likely to be important during stromal decidualization and awaits further study.

Frizzled-related protein (FRP) is a member of the Wnt family and is downregulated in the endometrium during cAMP treatment (Supplemental Table A) and during the window of implantation (28). Roles of the Wnt family in endometrial stromal cell function are currently under study in our laboratory.

Cyclin B and cyclin E2, cell cycle regulatory genes, were downregulated with cAMP treatment, suggesting that decreased expression of these mediators inhibits cell division in stromal decidualization. Cyclin B expression is regulated by progesterone in a breast cancer cell line (T47D-YB) (20), where progesterone had a biphasic effect on cell growth: initially accelerating breast cancer cells through the first mitotic cycle and then subsequently arresting them in late G_2 of the second cycle (20). The G_1 arrest was associated with decreased levels of cyclin D1, D3, and E, and disappearance of cyclin A and B, with induction of cyclic-dependent kinase inhibitors p21 and p27 (Kip1) (20). In concordance with progesterone's effects of stimulating initial cell replication and then promoting cell cycle arrest, the activity of the cell cycle-dependent protein kinase, cdk2, is regulated biphasically by progesterone: it increases initially, then decreases (20). A second treatment with progesterone cannot restart proliferation despite adequate levels of transcriptionally competent progesterone receptor. Instead, a second progesterone dose delays the fall of p21 and enhances the rise of p27 (Kip1), thereby intensifying the inhibition on cell cycle progression (20). It is postulated that the G_1 arrest after progesterone treatment is accompanied by cellular changes that permit other, possibly tissue-specific, factors to influence the final proliferative or differentiative state (20). Cyclin E2 is a cell cycle regulatory gene that associates with Cdk2 in a functional kinase complex and regulates the G_1/S transition (21). Overexpression of cyclin E2 in mammalian cells accelerates G_1, demonstrating that cyclin E2 may be rate limiting for G_1 progression (21).

Models of In Vitro Decidualization in Human Endometrial Stromal Cells

The traditional model of in vitro decidualization of the human endometrial stromal cell involves the treatment of stromal cells with progesterone after estrogen priming (1). The decidual morphologic phenotype has been described in several in vitro studies in the literature of treating endometrial stromal cells with activators of the PKA pathway, in the absence of other known decidualizing stimuli (6, 37, 39, 53). Several examples of the characteristic morphologic and secretory phenotype of the stromal cell upon treatment with cAMP in vitro (in the absence of E2/P4), have been published in the literature (6, 37, 39, 53), correlating with our current microarray study where we found evidence of the characteristic morphologic changes and secretory products (i.e., IGFBP-1) of the decidualized stromal cell after 48 h of treatment with cAMP.

Mizuno et al. (37) compared the treatment of human endometrial stromal cells with progesterone, medroxyprogesterone acetate (MPA), prostaglandin E_2, or 8-Br-cAMP, and found that treatment with 8-Br-cAMP resulted in the most rapid decidualization response and the highest level of differentiation (as measured by morphology and secretion of known products of decidualized stromal cells, i.e., IGFBP-1). In this study it

was found that both progesterone and MPA required more than 2 wk to induce decidualization in vitro (37). Based upon these results, the authors speculated that there are two independent signals in decidualization in vitro, a stronger and more rapid cAMP-mediated signaling pathway and a progesterone receptor-mediated signaling pathway (37).

The ability of cAMP to mediate in vitro decidualization independently of progesterone was demonstrated by Tang et al. (53). They demonstrated that 8-Br-cAMP induces prolactin secretion in human endometrial stromal cells in addition to provoking the differentiation of the fibroblast-like stromal cells to the decidualized phenotype, as evidenced by both morphologic changes and by the expression of the products characteristic of decidualized cells, e.g., IGFBP-1, desmin, hsp 27, and laminin (53).

A manuscript by Brar et al. (6) demonstrated the importance of cAMP for decidualization and that the effects of progesterone on decidualization may be mediated via the cAMP signaling pathway. Progesterone treatment of endometrial stromal cells resulted in increased cAMP levels that positively correlated with levels of prolactin secreted into the conditioned medium (53). In addition, all-*trans* retinoic acid, which attenuates progesterone-dependent decidualization, significantly decreased both cAMP levels and the secretion of prolactin by endometrial stromal cells treated with progesterone, possibly implicating that the effects of progesterone on decidualization are mediated via the cAMP pathway (54). In addition, the PKA inhibitor, 8-bromoadenosine-3′,5′-cyclic monophosphorothioate, significantly suppressed progesterone-dependent prolactin expression, also suggesting that progesterone's effects on decidualization are mediated through cAMP (55).

A recent manuscript by Yoshino et al. (58) describes progesterone's inhibition of the phosphorylation of AKT/PKB and that H-89, a PKA inhibitor, inhibits this effect, further supporting the PKA pathway as a mediator of progesterone's action in endometrial stromal cells.

Despite this strong evidence of the PKA pathway mediating decidualization of human endometrial stromal cells in vitro,

Table 8. *Patterns of temporal gene expression of selected genes of interest during induction of decidualization with cAMP by functional category*

Ontology Category	Early	Middle	Late
Cell growth genes	NOT	IGFBP-1	NOT (second peak)
	NGFI-B/nur77	TGF-β1	NGFI-B/nur77 (second peak)
	Platelet-derived growth factor-α receptor	TGF-β-2	Platelet-derived growth factor-α receptor (second peak)
		VEGF-receptor 2	**IGFBP-5**
		Carcinoembryonic antigen	Activin β_B-subunit precursor
		EBAF	
		Rb107	
		IGF II precursor	
		IGF I receptor	
		Platelet-derived growth factor receptor	
		human beta nerve growth factor	
Neuropeptides		Preprosomatostatin	
		Somatostatin receptor, sstr2	
		L5 orphan G-protein-coupled receptor	
Cell cycle	N-ras regulation/cancer	Rabaptin-4	N-ras (second peak)
		Cyclin G2	snoI
		Cyclin B	**Cyclin E2**
		FRP, frizzle-related protein	
Signal transduction	E2F heterodimeric protein	DDX 14, helicase-like protein	**Activates NF-κ B**
		SALL1 gene	**L-kynurenine hydrolase**
		PTH/PTHrP receptor	**Retinal short-chain dehydrogenase/reductase**
			PLA2, calcium dependent phospholipid binding protein
			Glutathione reductase
			Lipocortin III
Immune genes	**IL-2 receptor**	IL-11	**Human allograft inflammatory factor**
		Semaphorin III	
		FHR-2 complement receptor protein	
		Factor XIII precursor	
		NK receptor	
Structural proteins	**Fibronectin**	Prestromelysin (MMP-10)	**Actin/tropomyosin/calmodulin binding protein**
		Matrilysin	**α1 Type XVI collagen**
		Myosin heavy chain homolog	
		Complement cytolysis inhibitor	
Enzymes		Ligand for Tie2/Tek receptor tyrosine kinase	Cyclophilin-40
		MAP kinase phosphatase	Helicase-MOI
			Phenylalanine hydroxylase
			Glutamate dehydrogenase
			Aldehyde dehydrogenase
			Phospho*enol*pyruvate carboxykinase

Genes that are repressed are shown in bold. Time indicated as early (0–2 h), middle (12–24 h), and late (36–48 h) is the time of peak gene expression, relative to *time 0* during the time course studied.

this signaling pathway may not be the sole mechanism mediating progesterone-induced decidualization. While experimental evidence suggests that progesterone exhibits its effects on decidualization through the cAMP pathway (12, 53, 54, 58), progesterone may mediate its effects on decidualization through additional signal transduction pathways than cAMP. In our previous microarray study (8) on endometrial stromal cell decidualization in vitro in response to cAMP or progesterone (after estradiol priming), most genes were coordinately regulated by both treatments, strongly suggesting PKA as a mediator of progesterone action in the stromal cell. Although the PKA pathway may be a mediator of progesterone's induction of decidualization in the stromal cell, it may not be the only mechanism of action.

Phenotypic Changes upon Treatment with cAMP

The decidualized human endometrial stromal cell phenotype has been observed in several in vitro studies of treating endometrial stromal cells with activators of the PKA pathway (6, 37, 39, 53) in the absence of other decidualizing stimuli. These studies correlate with our current microarray study where we found evidence of the characteristic morphologic changes and secretory products (i.e., IGFBP-1, prolactin, hsp 27, laminin) of the human endometrial decidualized stromal cell after 48 h of treatment with cAMP. After 24 h of treatment with cAMP, it was noted that the stromal cells began a transformation from elongated spindle-like fibroblasts into polygonal epithelial-like cells. In parallel with the change in the morphologic phenotype of the stromal cells observed at 24 h of treatment with cAMP, the levels of IGFBP-1 protein measured to be secreted from the stromal cells increased markedly between 12 and 24 h. At 48 h, the time point of maximal IGFBP-1 secretion over the time course studied, we found a predominance of the cells treated with cAMP which had transformed into polygonal epithelial-like cells, characteristic of decidualized cell morphology. Many of the genes we found in our microarray which play a known or speculated role in mediating the morphologic or secretory changes during decidualization of the human endometrial stromal cell (i.e., IGFBP-1, prolactin, activin subunits, VEGF, IL-11, EBAF, and other TGF-β family members) also increased in expression markedly between 24 and 48 h of treatment with cAMP, correlating with the timing of the phenotypic changes observed during our time course (Table 8).

Model of Gene Ontology in Response to Endometrial Stromal Treatment with cAMP

The data presented herein support the hypothesis that activation of the PKA pathway in response to cAMP in the human endometrial stromal cell requires a complex pattern of gene regulation, leading to a combination of induction and repression of individual genes and support the model in Fig. 11. To differentiate into the secretory phenotype, the stromal cell must undergo genetic reprogramming (53) to promote cell cycle arrest and to replace existing genes regulating stromal cell differentiation with new genes to mediate the adoption of the unique phenotypic changes seen with decidualization. The induced genes are expected to play a role in the changes in metabolism, hormone production, secretory capacity, and paracrine functions of the decidualized stromal cell. The presence of NOT, NGFI-B/nur77, and N-ras that function in cell growth, differentiation, and cell cycle regulation, early in decidualization suggests that they mediate early cell growth and differentiation, which are subsequently repressed, with a separate repertoire of genes with other functions being induced subsequently and important in mediating the functions of cell growth and differentiation. Endometrial stromal cells may undergo endoreduplication in the earliest phase of decidualization, which could be stimulated by various cell cycle regulatory

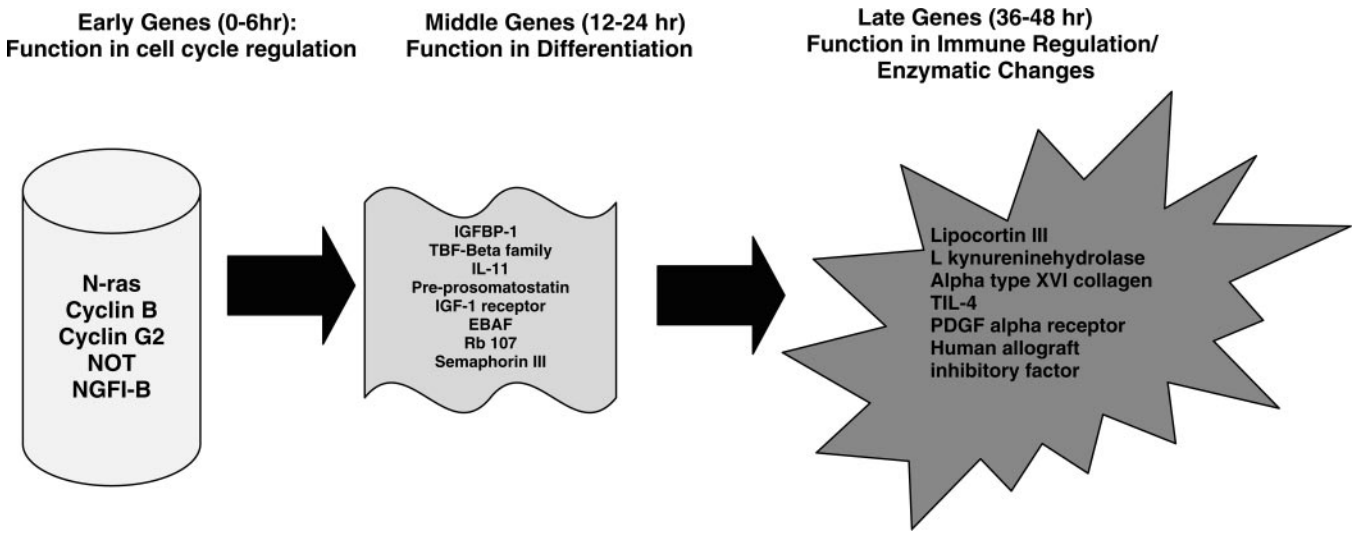


Fig. 11. Model of decidualization presented by microarray data. A cartoon model describing the gene expression underlying decidualization in three phases. Early in the response to cAMP (0–6 h) genes involved in cell cycle regulation are regulated that are postulated to mediate the early events of mitosis, endoreduplication, and subsequent cell cycle arrest. These events of early cell growth and cell division set the stage for the next set of events (12–48 h) in which the genes expressed are primarily involved in cellular differentiation, as the cell begins to both morphologically and functionally adopt the decidualized phenotype, including, middle genes (12–24 h), which are primarily involved in cellular differentiation and the adoption of the morphologic and secretory phenotype, and late genes (36–48 h), which mediate more specialized functions of the stromal cell, including immune regulation, signal transduction, and key enzymatic pathways.

genes [e.g., snoI, N-ras, NF1, Rb-related protein (p107), trk, Kip 2] and subsequently undergo an inhibition of cellular proliferation and induction of cellular differentiation. Subsequently, cell cycle arrest ensues, and genes implicated in stromal cell differentiation are induced at later time points, including IGFBP-1, preprosomatostatin, TGF-β1, IL-11, and EBAF. Somatostatin, the most significantly upregulated gene herein, may play a key role in regulating cellular proliferation and differentiation, acting as a molecular switch to inhibit decidual stromal cell proliferation and to promote the development of the stromal cell secretory phenotype, via regulation of IL-11, and angiogenesis via VEGF. Other factors likely contribute, as well.

Downregulation of TIL-4, known to act via the NF-κB pathway to stimulate the production of IL-1, IL-6, and IL-8, suggests that in the decidualized stromal cell it may play a key role in regulating these cytokines and as an immune modulator at the trophoblast:decidua interface. TNF-related apoptosis inducing ligand (TRAIL), found to be upregulated in our initial microarray (49) and confirmed herein, may play an important role in immune regulation in implantation, as it is a known activator of apoptosis in activated lymphocytes. In addition, remodeling of the extracellular matrix is an important role of the decidual cell. Thus many genes expressed temporally in response to cAMP are proposed to be important for autocrine regulation of stromal cell mitosis and differentiation and for paracrine communications with endogenous and transient cell populations present in nonpregnant endometrium in anticipation and during the initial stages of implantation.

Although the current study opens new opportunities to explore the function of a diverse group of gene families regulated by cAMP and in the events underlying decidualization, it should be noted that in vitro endometrial stromal cells may exhibit different patterns of behavior and gene expression under in vivo conditions where endocrine factors and/or paracrine interactions may affect decidual gene programming. Comparing patterns of gene expression in vitro and in vivo is critically important to validate these data, and additional validation with animal models is essential to provide insight into mechanisms underlying endometrial stromal cell decidualization and functions of the decidualized endometrial stromal cell during implantation as it communicates with the conceptus, immune cells, and other cell populations present during implantation. Derived functions should provide insight into endometrial disorders that have a major impact on women's reproductive health, normal pregnancy, and pregnancy disorders, as well as fetal growth and development. Candidate genes necessary for decidualization may also be utilized as diagnostic screens and targets for drug discovery in women with endometrial-based infertility, and identification of the molecular mechanisms underlying decidualization and implantation can lead to the development of novel contraceptives (Fig. 11).

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