BIOLOGY OF REPRODUCTION **76**, 102–117 (2007)
Published online before print 4 October 2006.
DOI 10.1095/biolreprod.106.054791

Decidual Stromal Cell Response to Paracrine Signals from the Trophoblast: Amplification of Immune and Angiogenic Modulators[1]

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ABSTRACT

During the invasive phase of implantation, trophoblasts and maternal decidual stromal cells secrete products that regulate trophoblast differentiation and migration into the maternal endometrium. Paracrine interactions between the extravillous trophoblast and the maternal decidua are important for successful embryonic implantation, including establishing the placental vasculature, anchoring the placenta to the uterine wall, and promoting the immunoacceptance of the fetal allograph. To our knowledge, global crosstalk between the trophoblast and the decidua has not been elucidated to date, and the present study used a functional genomics approach to investigate these paracrine interactions. Human endometrial stromal cells were decidualized with progesterone and further treated with conditioned media from human trophoblasts (TCM) or, as a control, with control conditioned media (CCM) from nondecidualized stromal cells for 0, 3, and 12 h. Total RNA was isolated and processed for analysis on whole-genome, high-density oligonucleotide arrays containing 54 600 genes. We found that 1374 genes were significantly upregulated and that 3443 genes were significantly downregulated after 12 h of coincubation of stromal cells with TCM, compared to CCM. Among the most upregulated genes were the chemokines *CXCL1* (*GRO1*) and *IL8*, *CXCR4*, and other genes involved in the immune response (*CCL8 [SCYA8]*, pentraxin 3 (*PTX3*), *IL6*, and interferon-regulated and -related genes) as well as *TNFAIP6* (*tumor necrosis factor alpha-induced protein 6*) and metalloproteinases (*MMP1*, *MMP10*, and *MMP14*). Among the downregulated genes were growth factors, e.g., *IGF1*, *FGF1*, *TGFB1*, and angiopoietin-1, and genes involved in Wnt signaling (*WNT4* and *FZD*). Real-time RT-PCR and ELISAs, as well as immunohistochemical analysis of human placental bed specimens, confirmed these data for representative genes of both up- and downregulated groups. The data demonstrate a significant induction of proinflammatory cytokines and chemokines, as well as angiogenic/static factors in decidualized endometrial stromal cells in response to trophoblast-secreted products. The data suggest that the trophoblast acts to alter the local immune environment of the decidua to facilitate the process of implantation and ensure an enriched cytokine/chemokine environment while limiting the mitotic activity of the stromal cells during the invasive phase of implantation.

decidua, gene regulation, immunology, implantation, trophoblast

[1]Supported by the German Research Foundation (Deutsche Forschungsgemeinschaft HE 3544/1 to A.P.H.) and by the NIH Cooperative Program in Trophoblast-Maternal Tissue Interactions U-01 HD-42280 (to A.T.F.), U-01 HD-42283 (to S.J.F.), and U-01 HD-42298 to (L.C.G.). All gene expression arrays and raw .CEL files have been deposited with the National Center for Biotechnology Information gene expression and hybridization array data repository (GEO, available at http://www.ncbi.nlm.nih.gov/geo/), GEO accession number GSE5809.
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Received: 21 June 2006.
First decision: 17 July 2006.
Accepted: 21 August 2006.
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ISSN: 0006-3363. http://www.biolreprod.org

INTRODUCTION

The major goals in establishing a successful pregnancy are to anchor the conceptus in the maternal uterine wall, establish a vascular supply to enable optimal growth and development of the conceptus, and protect the fetal allograft from rejection by the maternal immune system. To achieve these goals, there are complex molecular dialogues that take place among the maternal endometrium (decidua), the conceptus, and the placenta. After attachment and intrusion through the luminal epithelium, the invasive phase of implantation begins [1], lasting until approximately 20 wk of gestation [2, 3] and involving dynamic crosstalk between trophoblasts and cells in the maternal decidua and the endometrial extracellular matrix (ECM). These interactions are directed by genetic programming of specialized trophoblast phenotypes (invasive, endothelial, cytotrophoblasts, and syncytiotrophoblasts) [4] and various cell types in the decidua, including stromal fibroblasts, vascular endothelial cells, smooth muscle cells, and leukocytes [5], in the context of a defined steroid hormone milieu [6, 7]. Integrins, cytokines, chemokines, growth factors, matrix-degrading enzymes, their inhibitors, and others [5, 6, 8, 9] are believed to be involved, given observations in relevant knockout mouse models and in vitro studies and expression analyses. Direct trophoblast-decidual interactions, however, have yet to be defined.

Differentiation (decidualization) of endometrial stromal fibroblasts occurs in vivo in response to progesterone (P_4) [10, 11]. It begins in the periarteriolar regions and subepithelially, is histologically evident in fibroblasts in microenvironments throughout the stroma in nonconception cycles, and is more robust and complete in conception cycles [10]. The

decidua secretes products (e.g. fibronectin and insulin-like growth factor [IGF] binding protein-1 [IGFBP1]) that bind to trophoblast-specific integrins and modulate trophoblast migration, invasion, or both in in vitro models [12]. The invading trophoblast secretes matrix metalloproteinases (MMPs) that degrade the decidual ECM [2, 13, 14], and the maternal decidual stromal cells express high levels of the tissue inhibitors of MMP (TIMPs) [15], which also modulate trophoblast invasiveness in vitro [14, 16]. In addition, cytokines present at the placental-decidual interface, such as interleukin 1 beta (IL1B) and transforming growth factor beta (TGFB) [16], regulate trophoblast invasion in vitro through their effects on MMP and TIMP production, respectively [14, 17, 18]. The trophoblast products, IL1B and IGF2, inhibit TIMP3 and IGFBP1 expression in human decidualized endometrial stromal fibroblasts in vitro, suggesting that the trophoblast modulates its own invasiveness by regulating maternal restraints and facilitators of invasion in the decidua.

In addition to their complex interactions with trophoblasts, human decidualized endometrial stromal fibroblasts play an important role in orchestrating the repertoire and behaviors of immune cells within the decidua during early pregnancy [8, 19, 20]. Cytokines and chemokines largely mediate these interactions. The trophoblast must maintain its allogenic phenotype and avoid targeting by the maternal immune system. To this end, it also secretes cytokines and chemokines that attract peripheral and resident leukocytes in the maternal decidua, which, in turn, provide local immunomodulation and support placental development [21, 22]. Thus, the immune environment in the endometrium derives from endogenous immune cells and an influx of leukocytes from the peripheral circulation, with the products secreted from decidualized endometrial stromal fibroblasts, as well as trophoblasts, both putatively important in endogenous leukocyte proliferation, differentiation, recruitment, and trafficking into the endometrium in early pregnancy [7, 23, 24]. These immune cells, in turn, have cytokine repertoires that can facilitate the recruitment of additional immune cells as well as regulate their state of activation [8, 24]. The interactions between the trophoblasts and the decidual stromal fibroblasts that facilitate the achievement of these goals, however, are just beginning to be appreciated [7] and are likely of paramount importance in successful implantation, normal fetal growth and development, and healthy pregnancies.

To investigate paracrine communications that occur at the decidua-trophoblast interface, we studied the effects of products secreted from human trophoblasts on global gene expression in in vitro-decidualized human endometrial stromal cells, a model of the decidual phenotype in human pregnancy [10, 25, 26]. The data support a model of trophoblast modulation of decidualized stromal fibroblast production of immune and angiogenic factors that may regulate—directly or indirectly—trophoblast differentiation, invasion, and placentation as well as leukocyte recruitment, leukocyte proliferation, leukocyte activation, and immunoacceptance of the fetal allograft. Furthermore, the data support a role for the decidualized stromal fibroblast as a kingpin in the amplification of signals from the trophoblast to ensure an optimal immune and angiogenic milieu at the placental-decidual interface for successful implantation.

MATERIALS AND METHODS

Experimental Design

The goal of the present study was to investigate gene expression changes in decidualized human endometrial stromal cells, a model of the decidual cells in pregnancy, in response to the products secreted by invading human cytotrophoblasts in vitro. The experimental design is complex (Fig. 1) and is composed of 1) decidualization of human endometrial stromal cells in response to P_4; 2) culturing of invasive human cytotrophoblasts in vitro and harvesting the trophoblast-conditioned medium (TCM), which contains products secreted by these cells; 3) treating the decidualized human endometrial stromal cells with TCM; and 4) treating the decidualized human endometrial stromal cells with media conditioned by an irrelevant cell-type control conditioned media (CCM) or an unconditioned medium (pilot study). The section below describes the materials and methods used in these experiments.

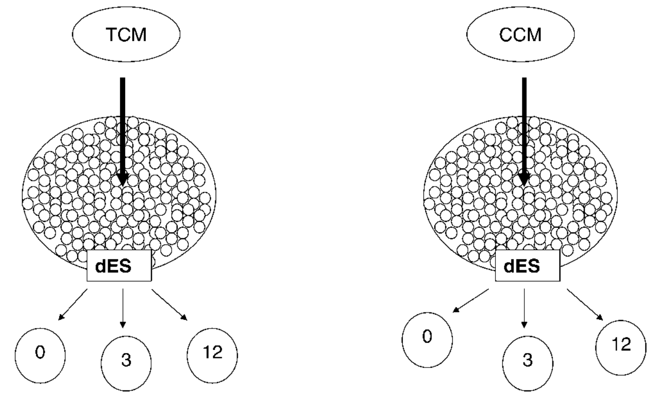

FIG. 1. Schematic diagram of experimental design (see text). Decidualized human endometrial stromal cells (dES) were treated with trophoblast-conditioned medium (TCM) ("experimental group") or control conditioned medium (CCM) ("control group") for 0, 3, or 12 h. At the conclusion of the treatments, cells were processed, and media were collected, as described in *Materials and Methods*.

Endometrial Tissue Specimens and Stromal Cell Culture

Endometrial samples were obtained from normally cycling women undergoing hysterectomy or endometrial biopsy for benign conditions after written informed consent and under an approved protocol by the Stanford University Committee on the Use of Human Subjects in Medical Research and the Committee on Human Research, University of California, San Francisco. Samples were obtained from n = 7 subjects, 26–46 yr old, with regular menstrual cycles (28–35 days), who had been documented not to be pregnant and not on steroid hormone medications within 3 mo of endometrial sampling. Endometrial tissue was subjected to collagenase/hyaluronidase (Sigma-Aldrich) digestion for 2 h at 37°C. After digestion, the stroma was dispersed, whereas the epithelial structures remained mostly intact. Stromal cells were separated from the epithelium on the basis of size, as previously described [27]. Stromal cells were centrifuged, and the resulting pellet was resuspended in a 4:1 ratio of Dulbecco modified Eagle medium DMEM-F12 (Gibco, Grand Island, NY) and DMEM with 10% v/v charcoal-stripped fetal bovine serum (FBS; Gibco). The cells were preplated in 10-cm^2 standard-culture plates (Costar, Corning, NY) for 1 h at 37°C and 9% CO_2, and the medium was replaced with serum-containing media containing high-glucose DMEM and molecular cell developmental biology media (DMEM/MCDB-105) with 10% v/v charcoal-stripped FBS, insulin (5 μg/ml; Sigma-Aldrich), gentamicin, penicillin, and streptomycin, as described [28]. Endometrial stromal cells were passaged two to three times prior to use in experiments. Purity was >99% by vimentin immunostaining [12].

Decidualization of Endometrial Stromal Cells In Vitro

Endometrial stromal cells from n = 3 different patients were plated on six-well tissue culture-grade plates (Corning). Decidualization was conducted in vitro by pretreating the cells with estradiol (E_2) (10 nM) in serum-containing media (as described above), and after reaching confluence, the cells were treated with E_2 + P_4 (E_2P_4) for 14 days in serum-free, decidualizing media (DM) containing high-glucose DMEM/MCDB-105 with ascorbic acid (50 ng/ml), transferrin (10 ng/ml), gentamicin (50 ng/ml), and E_2 (10 nM) + P_4 (1 μM). The concentration of IGFBP1 (a marker of stromal decidualization) in the media was determined after 14 days of E_2P_4 treatment by the total IGFBP1

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ELISA assay (DSL, Webster, TX), as described [29]. Cells with IGFBP1 levels ≥200 ng/ml in the media were considered decidualized [29].

Preparation of TCM and CCM

Trophoblast-conditioned media. Placentae were obtained from elective terminations of pregnancy (6–22 wk) after written informed consent under a protocol approved by the Committee on Human Research at the University of California, San Francisco, and the Stanford University Committee on the Use of Human Subjects in Medical Research. Placental tissue was collected within 1 h of isolation, washed thoroughly in PBS with antibiotics, and placed on ice. Cytotrophoblasts were isolated from first- or second-trimester human placentae, as described [18, 30]. Briefly, placentae were subjected to several enzymatic digests that result in the detachment of cytotrophoblast stem cells from the underlying stromal core of the chorionic villus. The detached cytotrophoblasts were then purified with a Percoll gradient and subsequently cultured on Matrigel-coated substrate (Collaborative Biomedical Products, Bedford, MA) at a concentration of 1×10^6 cells/ml in serum-free DM containing high-glucose DMEM/MCDB-105 with ascorbic acid (50 ng/ml), transferrin (10 ng/ml), gentamicin (50 ng/ml), E_2 (10 nM) + P_4 (1 mM) for 48 h at 37°C, and 5% CO_2. Cultures were >99% pure [30]. After 48 h, the cells were spun down, and the TCM was removed, pooled, and stored at −80°C until further use.

Control conditioned media. Because of the potential for an accumulation of cellular waste products in the TCM, the CCM was prepared by plating passaged, nondecidualized endometrial stromal cells at a density of 1×10^6 cells per 60-mm^2 well and culturing in DM for 48 h. After 48 h, the media were removed, pooled, and stored at −80°C until further use.

Incubation of Decidualized Endometrial Cells with TCM or CCM

Decidualized cells from n = 3 patients were incubated in six-well tissue culture-grade plates with 1 ml per well of either TCM or CCM for 3 and 12 h (Fig. 1). Each treatment was conducted in triplicate. RNA was extracted from cells at $t = 0$ h (no conditioned media incubation), $t = 3$ h, and $t = 12$ h of incubation with either TCM or CCM. Within each triplicate, cells from two wells were pooled for RNA isolation, and cells from one well were used for validation studies. An additional control was unconditioned media—i.e., DM that was added to decidualized stromal cells for 48 h—at the end of which the cells were processed for RNA isolation, as described above.

The time course of up to 12 h of exposure of decidualized endometrial stromal cells to TCM (and CCM) was based on a pilot experiment. In the pilot experiment, decidualized endometrial stromal cells were incubated with either TCM or DM for 12, 24, or 48 h. The most acute response of the decidualized stromal cells to the TCM treatment occurred at the 12-h time point (data not shown).

Microarray Procedures and Data Analysis

RNA extraction and microarray preparation. Total RNA was extracted from cells with Trizol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. The RNA preparations were then DNase treated and purified with the RNeasy kit (Qiagen, Valencia, CA). The purity and quality of the samples were determined by assessing their absorbance ratio at 260:280 nm and by an electrophoresis of the samples on 1.5% agarose gels. Samples were stored in RNase-free water until further use. With 2.5 μg of purified total RNA, samples were prepared for cRNA amplification and hybridization, as previously described [31]. Individual samples were hybridized overnight to high-density human genome arrays (HG U133 Plus 2.0; Affymetrix, Santa Clara, CA), containing 42 203 annotated genes and 12 397 expressed sequence tags (ESTs), at the Stanford University School of Medicine Protein and Nucleic Acid Facility. Subsequently, the chips were scanned with an HR3000 scanner, and the data were extracted by Affymetrix Gene-Chip Operating Software (GCOS) version 1.1.

Data Analysis

Microarray gene expression data analysis. The intensity values of different probe sets (genes) generated by the Affymetrix GCOS were imported into GeneSpring version 7.2 software (Agilent Technologies, Santa Clara, CA) for data analysis. The data files (.CEL files) containing the probe level intensities were processed by the robust multiarray analysis algorithm (GeneSpring) for background adjustment, normalization, and $\log_2$ transformation of perfect-match values [32]. Subsequently, the data were subjected to per chip and per gene normalization by GeneSpring normalization algorithms. Samples were subjected to a per chip normalization by taking the median expression value of all probe sets on a chip and dividing each gene expression value by this median value. Gene expression data from each TCM sample time point were then normalized to their corresponding CCM value for that time point, and finally, derived expression values were normalized to control samples at $t = 0$. We also analyzed the data without taking the CCM samples into consideration; the 3- and 12-h samples were directly compared to $T = 0$ h, and we got similar results. The resulting generated gene lists included only the genes that had a fold change value of 1.5 or higher and a P-value of <0.05 by a one-way ANOVA parametric test and a Benjamini-Hochberg multiple testing correction for false discovery rate. This combined gene list contains 3736 and 4818 probe sets differentially expressed at 3 h and 12 h, respectively.

Principal component analysis. Principal component analysis (PCA) is an unsupervised pattern recognition and visualization tool that is used to analyze large amounts of data derived from gene array expression analyses [33]. It displays a multidimensional data set in a reduced dimensionality (three dimensions) to maximally capture variations in the data. Each dimension represents a component to which a certain percentage of variance in the data is attributed. We applied the unbiased PCA algorithm in GeneSpring to all 14 samples of the TCM- and CCM-treated cultures and used all 54 600 probe sets on the HG U133 Plus 2.0.

K-means analysis. The differentially expressed genes from all pair-wise comparisons (combined gene list) were also subjected to K-means analysis [34] by GeneSpring software (Agilent Technologies). This analysis was used to determine kinetic patterns of gene expression analyzed across the time points t = 0, 3, and 12 h of exposure of decidualized endometrial stromal cells to TCM. K-means analysis was applied to the data by the smooth correlation of distance measure algorithm (GeneSpring), developed specifically for time-dependent samples with a clear separation of gene expression profiles. All differentially expressed genes were distributed among four clusters. This optimal clustering allowed all genes to be classified into these clusters, and no genes were unclassified. Gene expression values of members of each cluster group were averaged to show one profile for graphic representation of each cluster group.

GO classification. Differentially expressed genes at 3 and 12 h of culture with TCM versus controls, identified by microarray gene expression analysis, were queried for their respective gene ontology (GO) classes by the GOTree machine Web-based software from the Oak Ridge National Laboratory [35]. A Web-based tool, the Gene Ontology Tree Machine (GOTM) [35], was used to interpret biological, molecular, and cellular functions of genes identified in response to treatment with TCM. The GOTM uses GO hierarchies to discover significant biological processes, molecular functions, and cellular components in a gene list. It implements a statistical analysis of the GO categories for the input gene list and suggests biological areas of potential value in further investigation [36]. First, the differentially expressed genes are classified by their corresponding GO categories, and the observed number of genes in each of these GO categories is recorded. Genes represented on the HG U133 Plus 2.0 Affymetrix chip represent the reference gene list. The expected number of genes in each GO category corresponds to the number of genes falling into that GO category in the reference gene list. A given GO category is considered enriched when the observed number of genes in that category is greater than the expected number. The GO classifications for different conditions were derived, taking into account only the nonredundant and specific GO classes for each condition. Enriched GO classes and the percentage of regulated genes in each enriched group are presented in pie chart graphics in *Results*. All GO categories are available online at www.biolreprod.org and have been deposited at the GEO.

KEGG pathways. Genes that were differentially expressed in decidualized endometrial stromal cells treated for 3 and 12 h with TCM, compared to CCM, were classified in pathways. Hypergeometric testing was applied to derive the enriched genes in each pathway with a P-value that was <0.05 [35]. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were derived from the up- and downregulated gene sets for each time point as well as each K-means cluster. Only select pathways and processes are highlighted in *Results*. All data are available online at www.biolreprod.org and have been deposited at the GEO.

Microarray Validation

Real-time PCR. With the Omniscript Reverse Transcription Kit with a 1:1 ratio of Oligo $(dT)_{16–18}$ and random hexamers (Invitrogen, Carlsbad, CA), cDNA was generated from 1 μg of total RNA isolated from each sample. With 0.3 μM (each) primer, real-time PCR was performed in triplicate in 25-μl reactions with the QuantiTect SYBR Green PCR Kit (Qiagen), according to the manufacturer's instructions. PCR primers were designed to be intron spanning (sense and antisense primers were positioned in different exons to avoid amplification of contaminating genomic DNA and to produce amplicons ranging from 131 to 318 bp [Table 1]). PCR reactions were run by the Mx4000 Q-PCR system (Stratagene, La Jolla, CA), and data were collected and

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TABLE 1. Primer pairs used for real-time PCR validation.

Gene	Forward Primer	Reverse Primer	Amplicon (bp)
RPL19	GTAAGCGGAAGGGTACAGCCA	TTGTCTGCCTTCAGCTTGTG	211
PTGES	TTTTCAGACTGACCCCAGAAA	TGAGCCAGATTGTACCACTTCA	210
ICAM1	CCCACCATGAGGACATACAAC	GGCCTTTGTGTTTTGATGCTA	260
MMP1	ATGGATCCAGGTTATCCCAAA	TCCTGCAGTTGAACCAGCTAT	270
TNFAIP6	TGGCTTTGTGGGAAGATACTG	TTTTCCTTGACTGGATTTGGA	260
TNFAIP3	TTTTGGGACTCCAGAAAACAA	CTTGAGGTGCTTTGTGTGGTT	297
IL8	GAAGAGAGCTCTGTCTGGAC	TCACTGGCATCTTCACTGAT	131
CCL8	ACAACCCAGAAACCTTCACCT	GGTGATTGGAATGGAAACTGA	136
PTX3	GCAGGTTGTGAAACAGCTAT	GTTTAATACATCTGTGGCT	132
CXCL1	ATAGCCACACTCAAGAATG	TCTGCAGCTGTGTCTCTCTT	194
IGFBP5	TGCACCTGAGATGAGACAGG	GCTTCATCCCGTACTTGTCC	215
ANGPT1	GGGGGAGGTTGGACTGTAATA	TTCCCTTCCCAGTCCATTAAC	188
FGF1	GCCCTGACCGAGAAGTTTAAT	AGCCTTTCCAGGAACAAACAT	174
SLC2A8	GTCTCTGCACAGCCTGTTGAT	AGGCTCCATAGGGCCTGAG	247
TGFB1	TCAGGAAAGAGGGGATGAACT	TGCATTCCTCCTGTAGTGCTT	161
FZD2	AGCGGCCTATCATTTTTCTGT	GCCAGGTGGAAGTACTGAGAG	295
CSRP1	AAATTTGCCCAGAAGATTGGT	AATGGAATGGCGATGAAAAG	318
IGF1	TCCTGCATCTCTTCTACCTG	CTCCAGCCTCCTTAGATCACA	234
KLF4	CGGCAAAACCTACACAAAGAG	TCTTCATGTGTAAGGCGAGGT	221

analyzed by Mx4000 software, as previously described [31]. The PCR amplification efficiency for each primer pair was estimated by conducting real-time PCR on serial dilutions of template. The amplification efficiency was calculated from the resulting standard curve by the following equation: $EFF = 10^{[-1/slope]} - 1$, as described [31]. Ct values were calculated by the Mx4000 software, based on fluorescence intensity values after normalization with an internal reference dye and baseline correction. The templates for both the gene of interest and the normalizer were diluted 4-fold from the real-time product for use in the PCR reaction. Fold change values were calculated by first normalizing them to $t = 0$ and then comparing them to the CCM. Statistical analysis was performed by the relative expression software tool (REST) [37].

Protein Expression

ELISA evaluations. We chose two of the most highly upregulated genes (*CXCL1* [*GRO1*] and *IL8*) for protein validation by ELISA. For both assays, cell culture supernatant (~1000 μl) was removed from the sample wells, at $t = 0$ of treatment and at the appropriate time interval (3 or 12 h), and diluted in the appropriate diluents for each respective assay. The CXCL1 ELISA was performed with a monoclonal anti-human CXCL1 antibody (R&D Systems, Minneapolis, MN). One hundred microliters per well of primary antibody (at a concentration of 4 μg/ml) was coated overnight onto 96-well Maxisorp plates (Nalge Nunc International, Rochester, NY). Plates were washed with wash buffer (1× PBS + 0.05% v/v Tween-20) and then blocked for 1 h at room temperature with blocking buffer (1× PBS + 1% w/v BSA, 5% w/v sucrose, and 0.05% w/v NaN_3). Serial dilutions of recombinant CXCL1 (R&D Systems), ranging from 60 pg/ml to 4 ng/ml, were used as the standard. One hundred microliters of standard or appropriately diluted culture supernatant was added to each well and incubated for 2 h at room temperature. After washing, 100 μl of biotinylated anti-human CXCL1 at a concentration of 200 ng/ml was used to complete the sandwich assay and was added to each well for 2 h at room temperature. A streptavidin-HRP conjugate was then added to the plate and incubated for 20 min at 25°C. The color was developed with a tetramethylbenzidine substrate solution, and the absorbance was measured at 450 nm.

Interleukin 8 (IL8) was assayed in the cell culture supernatant with the Endogen Human IL8 ELISA Kit (Pierce Biotechnology, Rockford, IL). Serial dilutions of the IL8 standard ranged from 25.6 pg/ml to 1.0 ng/ml.

The mean ± SEM for the n = 3 replicates was calculated. Paired *t*-test analyses were performed to determine the statistical significance between the TCM and CCM treatments, as well as between $t = 0$ and the respective treatment conditions (CCM and TCM).

Immunohistochemistry of select proteins. Proteins that corresponded to three highly expressed genes in decidualized human endometrial stromal cells treated with TCM for 3 and 12 h, compared to controls, were evaluated by immunohistochemistry, with archived human implantation sites obtained from elective terminations of first-trimester pregnancies (n = 3) under approved protocols at the Cook County Hospital and the University of Illinois at Chicago Institutional Review Boards. The gene products assayed were CCL8 (MCP2), CXCL1, and rabbit anti-human prostaglandin E synthase (PTGES). The rabbit anti-human CCL8 (MCP2) antibody was purchased from Chemicon (Temecula, CA). The goat anti-human CXCL1 antibody was purchased from R&D Systems. The PTGES antibody was purchased from Cayman Chemical (Ann Arbor, MI). Tissues were fixed in 10% buffered formalin and subsequently embedded in paraffin. Sections were cut at 5 μm and mounted onto Superfrost Plus microscope slides (Fisher Scientific, Hanover Park, IL). Sections were dried on a slide warmer overnight. Sections were deparaffinized in two changes of xylene, rehydrated in graded ethanol concentrations, and rinsed in Tris-buffered saline. Tissues that were stained for CXCL1 or PTGES were subjected to antigen retrieval in sodium citrate buffer (pH 6.0) in a pressure cooker for 5 min. All slides were quenched in 0.3% v/v H_2O_2 in methanol for 15 min. Immunostaining was done with the Vectastain Elite Kits (Vector Laboratories, Burlingame, VT). Briefly, sections were blocked in 3% v/v normal serum and incubated in primary antiserum overnight at 4°C. CCL8 was used at a concentration of 4 μg/ml. CXCL1 was used at a concentration of 10 μg/ml. The PTGES was used at a concentration of 4 μg/ml. Sections were then incubated for 30 min at room temperature with their respective biotinylated secondary antibodies (1:200). Sections were washed and incubated with avidin-biotin complex for 30 min at room temperature. Staining was visualized by immersing sections in 3,3′-diaminobenzidine tetra hydrochloride solution containing 0.03% v/v H_2O_2. Nuclei were counterstained with Gill hematoxylin. Slides were dehydrated and coverslipped. Slides were analyzed and photographed with a Nikon microscope and Spot Digital camera and software.

RESULTS

PCA, Expression Profiles, and K-Means Analysis

The PCA of the expression profiles with all of the genes and ESTs on the Affymetrix gene chip demonstrates a clear segregation of the samples into two main clusters, corresponding to the TCM and CCM groups (Supplement 1A, available at www.biolreprod.org). This unbiased approach demonstrates that the endometrial stromal cell gene expression in the samples treated with TCM at the two time points differs from the gene expression in the samples treated with the CCM at time zero. In addition, profiles of the normalized data for all genes and all experiments for decidualized endometrial stromal cell samples treated with TCM or CCM demonstrated marked changes in gene regulation at $t = 3$ h of treatment with TCM, with even more pronounced changes at 12 h (Supplement 1B, available at www.biolreprod.org). To determine the kinetics of gene expression in response to TCM, we used the K-means clustering approach, a method of identifying sets of genes whose expression is regulated in a similar way across different experimental conditions and a particularly useful way of identifying genes with similar regulation across a time course. K-means analysis applied to differentially expressed genes from all pair-wise comparisons for all samples revealed four

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TABLE 2. Most highly up- and downregulated genes by TCM.

Upregulated	Downregulated
CXCL1, CXCL2 (*GRO1, GRO2*)	*IGFBP5*
IL6	TRH degrading enzyme
IL8	*CD24, 24A*
PTX3	Angiopoietin 1
MMP1	*IGF1*
CCL2 (*MCP1*), *CCL8* (*MCP2, SCYA8*)	*INTGA4*
TNFAIP-2,-3,-6	*FGF1*
TNFAIP3 interacting protein	α-smooth muscle actin
CXCR4	Cystatin
IL2RA, IL7R, IL13A2, IL15R	Fibronectin 1
IFI-30,-35,-44,T4	*TGFB1*
IFNGR1	*CCNE2*
INFα-IP-15	Calpain
IRF1	Trophinin
Metallothioneins 1F,1E,1H,1L,1X, 2A	*CTGF*
JUN	*PI3K p85a*
Plasminogen activator, urokinase	*FZD2*
MMP-3,-10,-12,-14, ADAMTS7	*WNT4*
SOD2	
GOS2	
GAS1,GAS7	
IER3	
PDE3B	
4B WTAP	
Stanniocalcin	
S100A3	

major cluster groups that are distinguishable by the following characteristics: cluster 1, upregulated at $t = 3$ h and further upregulated at $t = 12$ h; cluster 2, upregulated at $t = 3$ h and downregulated at 12 h; cluster 3, downregulated at 3 h and upregulated at 12 h; and cluster 4, downregulated at 3 h and further downregulated at 12 h. Complete information for K-means clusters is in Supplement 1C, which is available at www.biolreprod.org. The focus of the present study is on genes, GOs, and pathways upregulated at 3 and 12 h and downregulated at 3 and 12 h. In addition to the figures and tables presented in *Results* and as supplemental data, all gene expression arrays and raw .CEL files have been deposited with the National Center for Biotechnology Information gene expression and hybridization array data repository (GEO, http://www.ncbi.nlm.nih.gov/geo/), GEO accession number GSE5809.

Endometrial Stromal Cell Genes Regulated in Response to 3 and 12 h of TCM Treatment

Pair-wise comparisons. Pair-wise analysis of genes regulated after TCM treatment, compared to the control (CCM), revealed that at 3 h (early response), there were 1251 upregulated and 2485 downregulated genes and that at 12 h (later response), there were 1374 upregulated and 3443 downregulated genes by a one-way ANOVA, with $P \leq 0.05$. At both time points, the most highly upregulated genes included (Table 2) *CXCL1* [*GRO1*], *IL6, IL8,* pentraxin 3 (*PTX3*), *CCL8* [*MCP2*], *tumor necrosis factor alpha-induced protein 6 (TNFAIP6), CXCR4*, and interferon (IFN)-induced and -regulated genes. The most highly downregulated genes included the inhibitor of DNA binding 3 (*ID3*), *IGFBP5*, fibronectin-1, angiopoietin-1, and *IGF1*. The complete gene lists for pair-wise comparisons of TCM-treated cells versus controls at each time point, compared to $t = 0$, are presented in Supplement 2A, which is available online at www.biolreprod.org. (Similar results were obtained with unconditioned medium as the control [data not shown], suggesting that spent medium is not causative of the stromal cell response.) Also, when we conducted the analysis of just TCM-treated cells at $t = 3$ h and $t = 12$ h compared to $t = 0$ h (i.e., not compared to CCM controls at each time point), the gene lists were very similar, with the highest up- and downregulated genes being identical, thus supporting the control group as being a valid control. Lists are sorted in descending order for genes regulated at 3 h and are also sorted in descending order for genes regulated at 12 h. Extensive bioinformatics analysis further revealed genes commonly up- or downregulated at 3 and 12 h and those that were uniquely up- or downregulated at these two time points. These data are presented in their entirety in Supplement 2B available online at www.biolreprod.org. The GOs and KEGG pathways for the up- and downregulated genes, as well as for the regulated genes and gene families, are presented below.

GO Classifications

Upregulated. GO classifications, including biological processes and molecular functions, for statistically significant upregulated genes at 3 and 12 h are shown in Figure 2. The immune response category comprises 14.8% of genes (Fig. 2A, upper panel) and includes cytokine-related genes, certain IFN-induced genes, and genes involved in antigen presentation. Other biological processes include genes involved in the negative regulation of cell proliferation, apoptosis, the I-κB/nuclear factor (NF)-κB kinase cascade, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) cascade, angiogenesis, and hemopoiesis. Relevant molecular functions in this group include (Fig. 2A, lower panel) transcription regulator activity, peptidase activity, cytokine activity/binding, IFN receptor activity, growth factor activity/binding, plasminogen activator activity, and transmembrane receptor protein (tyrosine) kinase activity. Note that GO categories reflect enrichment of genes involved in particular biological processes or molecular functions but that they do not reflect the magnitude of regulation of individual genes. Fold changes in specific genes and gene families are presented later in *Results*.

After 3 h of treatment with TCM, GO analysis revealed that the biological processes included genes involved in transcription, intracellular signaling cascades, and, interestingly, the notch signaling pathway, as well as cell cycle arrest, among others (Supplement 3A, pie chart, upper panel, available online at www.biolreprod.org). Relevant molecular functions (Supplement 3A, pie chart, lower panel, available online at www.biolreprod.org) include, among others, protein binding, TGFB and IGF binding and receptor activities, interleukin binding/receptor activity, and zinc ion binding. Thus, early in the response to TCM, decidualized stromal cells upregulate cytokine signaling.

At 12 h of treatment with TCM, several GO processes involving genes that are uniquely upregulated include the categories of (Supplement 3B, pie chart, upper panel, available online at www.biolreprod.org) antigen processing, transport of inorganic ions, and notch receptor processing. The more prevalent genes were those involved in signal transduction and intracellular signaling cascades. Molecular functions corresponding to genes uniquely upregulated at 12 h (Supplement 3B, lower panel, available online at www.biolreprod.org) include insulin receptor substrate binding and transferase, phosphotransferase, and kinase activities as well as receptor signal, insulin receptor substrate binding, and hematopoietin/IFN class and cytokine receptor signal transduction.

Downregulated. Biological processes involving downregulated genes at 3 and 12 h are shown in Figure 2B, pie chart, upper panel. These include genes involved in transcription and

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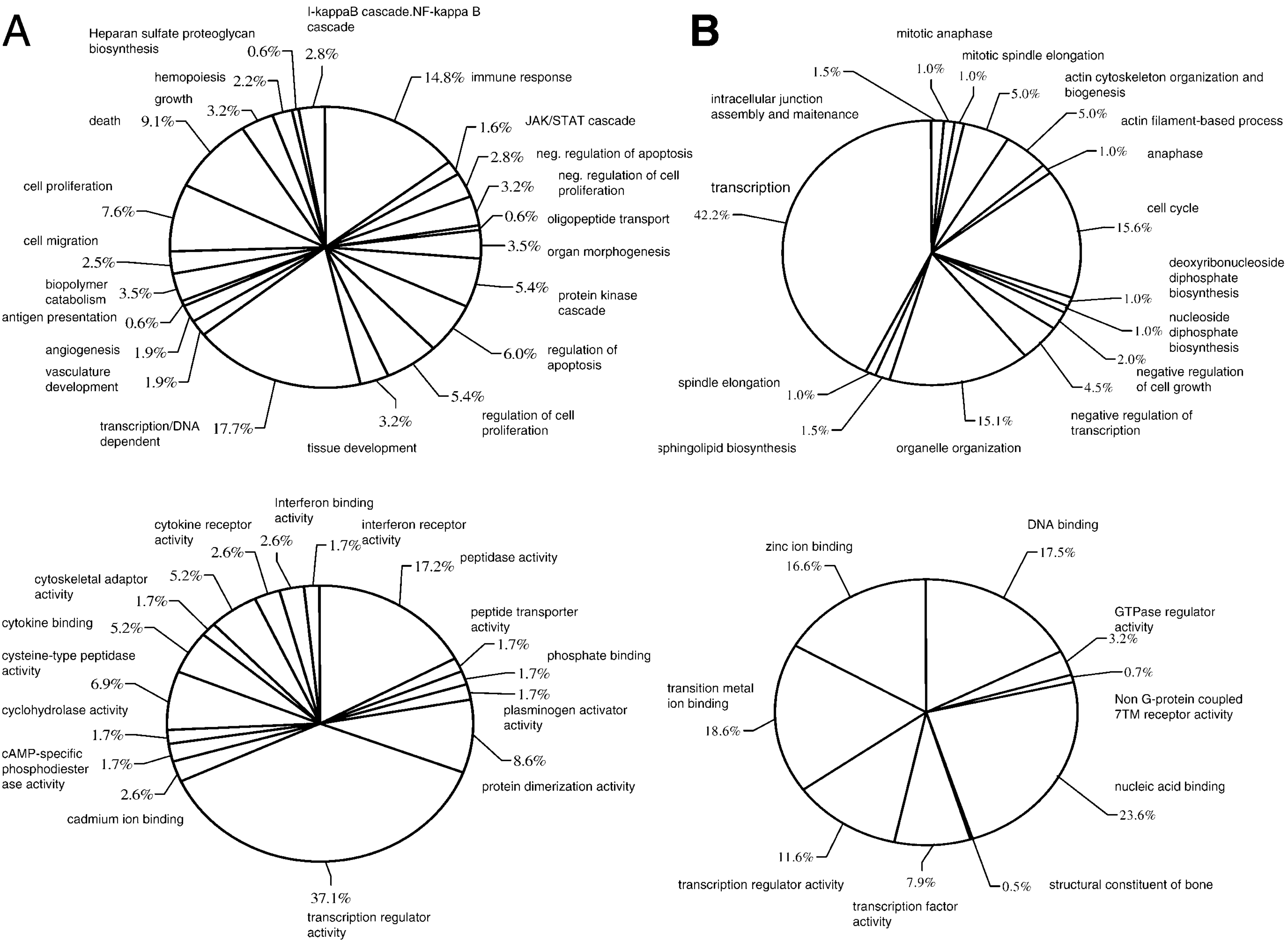

FIG. 2. Gene ontology (GO) classifications of biological processes and molecular functions for genes regulated in decidualized human endometrial stromal cells treated with TCM compared to controls, (**A**) for genes upregulated at 3 and 12 h and (**B**) for downregulated genes at 3 and 12 h. The pie charts show, for each condition, the enriched GO classes and the percentage of regulated genes that are in each class. Biological processes are shown in the upper panels, and molecular functions are shown in the lower panels. GO classifications for genes up- and downregulated at 3 or 12 h are shown in Supplements 3 and 4, available online at www.biolreprod.org, and have been deposited at the GEO.

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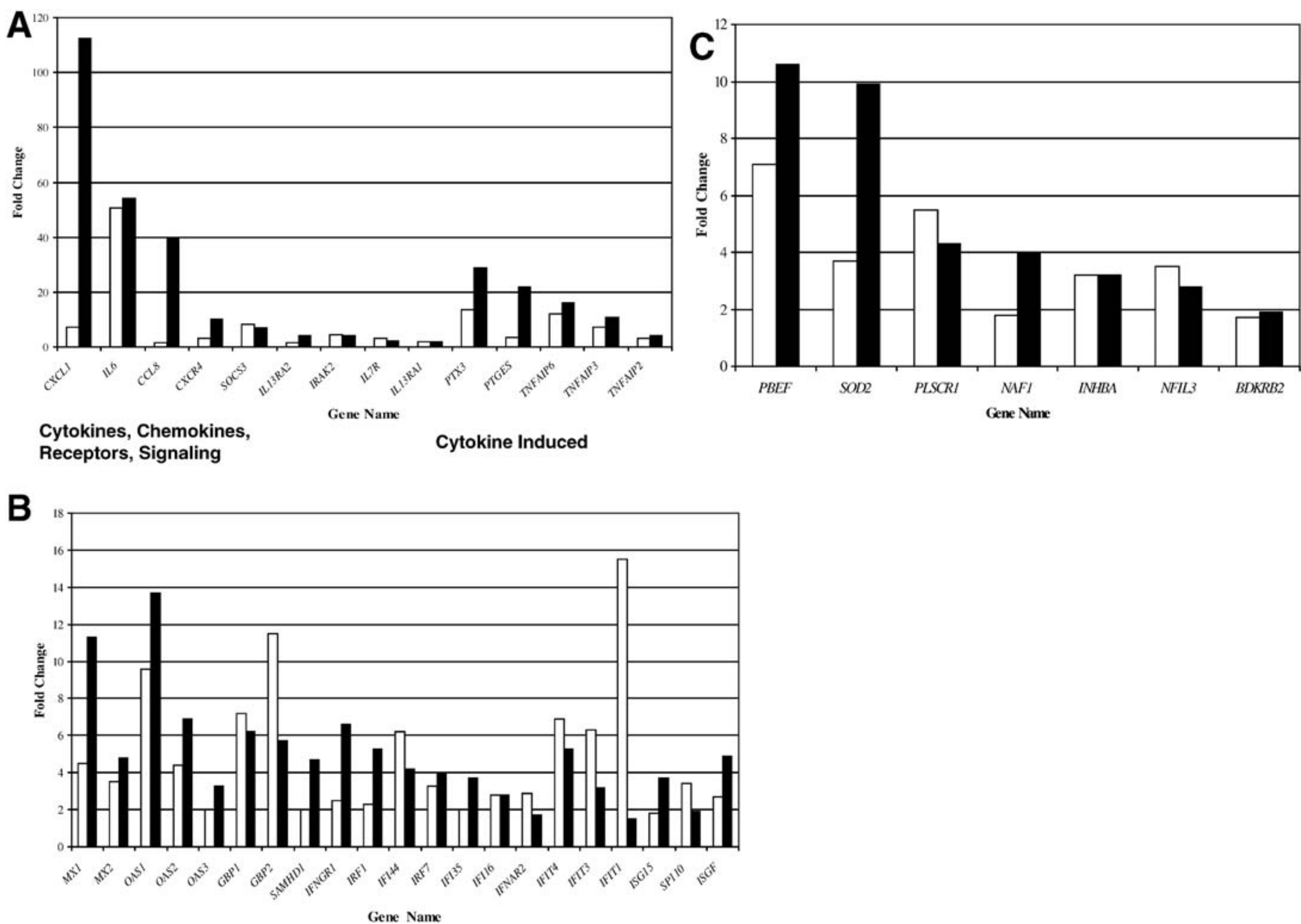

FIG. 3. Immune genes upregulated in decidualized human endometrial stromal cells after at 3 and 12 h of treatment with TCM, compared to controls; (**A**) cytokine and related immune genes; (**B**) interferon (IFN)-related or -induced genes; and (**C**) other immune-related genes. Gene names (abbreviations) are shown on the x-axis. The fold change is shown on the y-axis. White bars correspond to the fold change at 3 h, and black bars correspond to the fold change at 12 h.

actin cytoskeleton and actin filament basic processes, as well as the cell cycle processes. Molecular functions of downregulated genes include DNA and nucleic acid binding, transcription factor activity, and transition and zinc ion binding (Fig. 2B, pie chart, lower panel). These results suggest that an inhibition of endometrial cell mitosis takes place that is sustained during the 12 h of TCM treatment. Furthermore, genes involved in cellular metabolism and transcription are downregulated uniquely at 3 h, among others (Supplement 4A, available online at www.biolreprod.org). At 12 h, there is an inhibition of genes involved in cell adhesion, cell development, and cellular differentiation, as well as steroid hormone receptor signaling and protein binding and others (Supplement 4B, available online at www.biolreprod.org).

Commonly and Uniquely Upregulated Genes after 3 and 12 h of TCM Treatment

Upregulated at 3 and 12 h. Genes that are most highly upregulated at 3 h and continuing through 12 h of treatment with TCM, compared to controls, involve the immune response processes (Fig. 3). TCM stimulates the expression of genes that encode cytokines, chemokines, and their receptors and signaling pathway members, as well as genes that are cytokine induced (Fig. 3A). The most highly upregulated gene is *CXCL1* (*GRO1* or *GRO*α), which is a potent chemokine and angiogenic factor [38]. The most highly upregulated gene at 3 h, with upregulation sustained after 12 h of TCM treatment, is *IL6* (Fig. 3A). Others include *IL8*, important in leukocyte recruitment; *CXCR4*, a receptor for specific CXC ligands; *PTX3*, induced by IL1B and tumor necrosis factor beta (TNFβ) and a proinflammatory cytokine; *PTGES*, a proinflammatory cytokine induced by IL1B; and a group of TNF-induced proteins, including *TNFAIP6* and *TNFAIP2*, *TNFAIP3*, *TNFAIP8*, and *IL1A*. Although *IL1B* was upregulated at 3 h, the 12-h time points were not informative. Thus, overall, TCM induces predominantly a proinflammatory state at 3 h of treatment that is sustained at 12 h.

There is also upregulation of IFN-induced or -related genes (Fig. 3B), including genes involved in the IFN response (e.g., *MX1*, *MX2*, IFN-induced protein with tetratricopeptide (*IFIT*) repeats-1, −3, and −4), members of the 2′,5′-oligoadenylate synthetase (*OAS*) family, guanyl-binding proteins (*GBPs*), several IFN-induced proteins (*IFI16*, *IFI35*, and *IFI44*), and regulatory factors, as well as *JAK2*, which is involved in IFN signaling (Fig. 4A). Other upregulated immune-related genes (Fig. 3C) include pre-b-cell colony-enhancing factor (*PBEF*) and superoxide dismutase, *TAP1* and *TAP2* (transporters of antigens from the cytoplasm to the endoplasmic reticulum) (Supplement 2B, available online at www.biolreprod.org).

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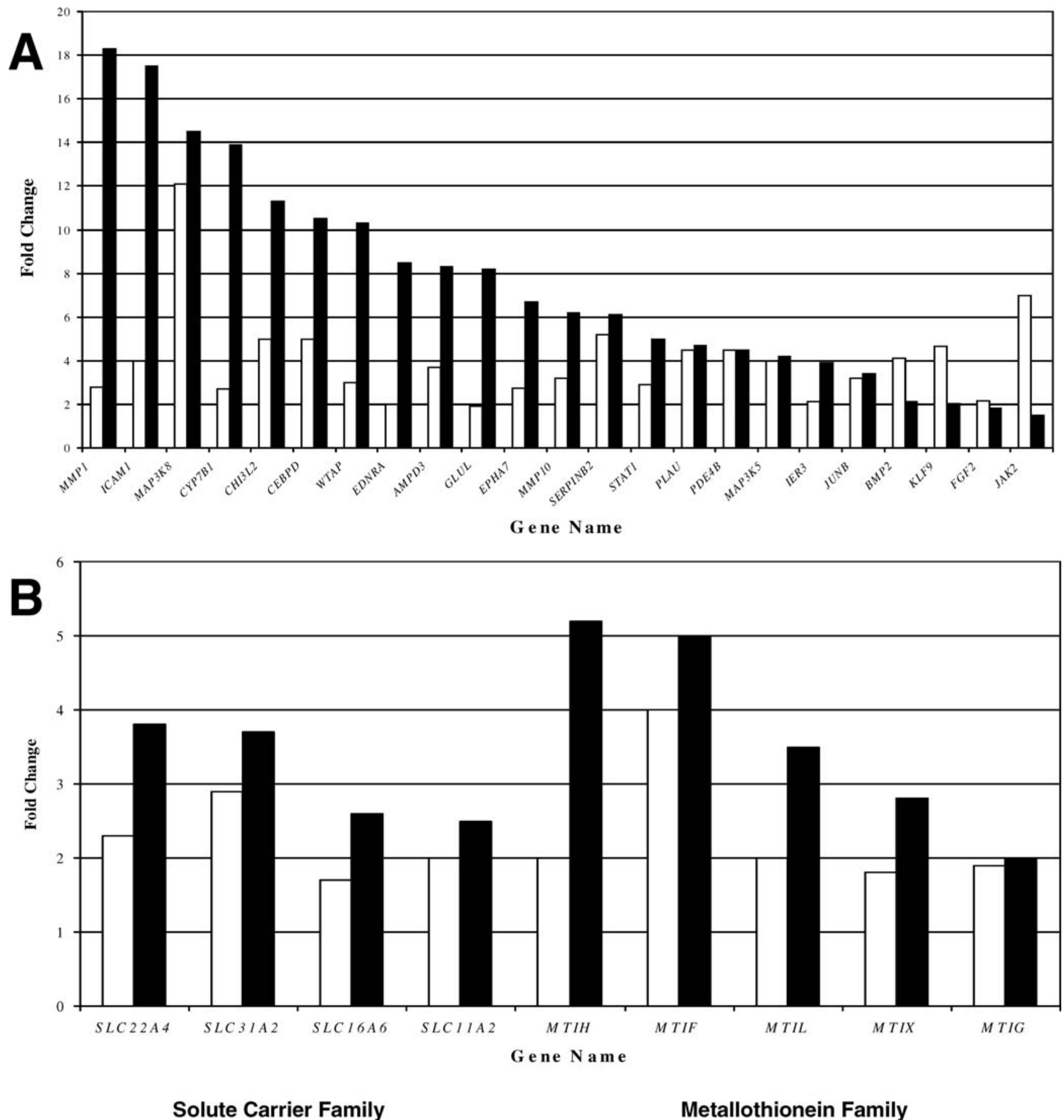

FIG. 4. Other genes (panel A) and gene families (panel B) upregulated in decidualized human endometrial stromal cells after at 3 and 12 h of treatment with TCM, compared to controls. Gene names (abbreviations) are shown on the x-axis. The fold change is shown on the y-axis. White bars correspond to the fold change at 3 h, and black bars correspond to the fold change at 12 h.

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Although the greatest number and magnitude of upregulated genes involve those participating in the immune response, other genes and gene families are also upregulated at 3 and 12 h after TCM treatment of endometrial stromal cells (Fig. 4). Examples include (Fig. 4A) the matrix-degrading enzymes, *MMP1* and *MMP10*, and urokinase (*PLAU*), a serine protease upregulated by IL1B. *PLAU* is also believed to play a role in angiogenesis and trophoblast invasion [39]. Interestingly, members of the *SERPIN* family, potent inhibitors of serine protease activity, are also upregulated. Additional genes upregulated at 3 and 12 h of TCM treatment include *ICAM1*, signaling components (e.g., members of the mitogen-activated protein [MAP] kinase family, *PDE4B*, *JUNB*, *JAK2*, *STAT1*), the endothelin A receptor, *BMP2*, and the oncostatin M receptor (*OSMR*) (an inhibitor of cytokine signaling), as well as numerous members of the metallothionein and solute carrier families (Fig. 4B).

Upregulated only at 3 h. Some genes are exclusively regulated at 3 h but not at 12 h (Table 3 and Supplement 2B, available online at www.biolreprod.org). These include the

TABLE 3. Genes uniquely regulated in decidualized human endometrial stromal cells after 3 or 12 h of TCM treatment.

Upregulated		Downregulated	
3 hours	**12 hours**	**3 hours**	**12 hours**
IL1B (4.30)	*PLD1* (5.47)	Zinc Finger Proteins	*CD24* (0.03)
IL15 (2.20)	*PDE3B* (5.35)	*ZNF177* (0.15)	*CD234A* (0.11)
PTGER4 (4.70)	*CD44* (3.92)	*ZNF91* (0.14)	Collagen Family
KLF5 (5.79)	*APOE* (2.75)	*ZNF42* (0.16)	*COL14A1* (0.07)
HGF (5.80)	*STAT3* (2.34)	*ZNF83* (0.36)	*COL12A1* (0.30)
PAPPA (4.60)	Solute Carrier Family	*ZNF555* (0.36)	*COL4A5* (0.37)
IRS2 (3.10)	*SLC39A8* (10.18)	*ZNF502* (0.36)	*COL5A1* (0.44)
EGFR (2.0)	*SLC16A3* (5.17)	Syndecan 1 (0.07)	*GATA6* (0.12)
IGF1R (2.70)	*SLC43A3* (5.11)	Toll-like Receptor (0.11)	*ANGPT1* (0.13)
KIT ligand (4.99)	*SLC11A2* (2.01)	*HOXA11* (0.31)	*GPR105* (0.15)
RIPK2 (4.45)	MMP's	Solute Carrier Family	Wnt-Related Genes
CDKN2C (2.52)	*MMP14* (4.05)	*SLC16A3* (0.38)	*SFRP1* (0.30)
CDKN1B (3.06)	*MMP12* (2.42)	*SLC12A6* (0.45)	*WNT5B* (0.38)
BCL3 (2.40)	*MMP3* (3.12)	*SLC25A16* (0.47)	Growth Factors
	HLA's	Collagen Family	*TGFB1* (0.24)
	HLA-C (2.24)	*COL13A1* (0.36)	*IGF1* (0.17)
	HLA-E (2.02)	COL22A1 (0.39)	KLF's
	HLA-F (2.07)	*Calmodulin* 2 (0.37)	*KLF4* (0.11)
	HLA-G (1.90)	*LIMD1* (0.41)	*KLF8* (0.14)
		CYR61 (0.46)	*KLF7* (0.14)
		ANGPT2	*HSD17B2* (0.31)
			TIMP3 (0.34)
			VEGF (0.43)
			JAK1 (0.24)
			CRSP1 (0.27)
			EGR3 (0.11)

Kruppel-like factor 5 (*KLF5*), a transcription factor important in angiogenic remodeling, and the hepatocyte growth factor (*HGF*), important in angiogenesis and morphogenesis [40]; the KIT ligand; the prostaglandin E receptor; *PAPPA*, the IGFBP4 protease; and the epidermal growth factor receptor (*EGFR)*, the *IGFIR*, and the *IRS2*. The latter genes suggest that the insulin/IGF receptor and EGF signaling are enhanced initially in response to TCM.

Upregulated only at 12 h. Genes that are upregulated uniquely at 12 h include members of solute carrier families, phospholipase D1, those involved in antigen presentation (*IFI30* and cathepsin S), intracellular signaling (*PDE3B* and *STAT3*), and *CD44*, which binds hyaluronan and is involved in leukocyte adhesion to endothelial cells in the ECM. In addition, two nonclassical major histocompatibility complex (MHC) molecules are upregulated: humor leukocyte antigen E (*HLA-E,* functioning to inhibit NK cytotoxicity) and *HLA-F* (known to be produced by the placenta). Also, *MMP3*, *MMP12*, and *MMP14* are uniquely upregulated at 12 h.

KEGG pathways of upregulated genes. Supplement 5A (available online at www.biolreprod.org) lists several pathways and the number of genes that are upregulated at 3 and 12 h of treating decidualized endometrial stromal cells with TCM. The greatest numbers of genes are involved in cytokine-cytokine receptor interactions, followed by the JAK-STAT and MAP kinase signaling pathways, axon guidance, NK-mediated cytotoxicity, and apoptosis. In addition, upregulation was observed of genes involved in the regulation of actin cytoskeleton, toll-like receptor signaling, and leukocyte trans-endothelial migration, among others. These pathways are also the predominant pathways (different genes represented in them) at 3 or 12 h of TCM treatment, with the exception of genes involved in focal adhesion being predominant at 3 or 12 h (Supplement 5A**,** available online at www.biolreprod.org).

Commonly and Uniquely Downregulated Genes after 3 and 12 h of TCM Treatment

Downregulated at 3 and 12 h. Some of the most highly downregulated genes at 3 and 12 h are shown in Figure 5. The most highly downregulated gene at both time points is the *ID3*. Others include LIM domain kinase 1 and several growth/transcription factors, such as *FGF1*, *SOX4*, *HOXA10*, and pre-b-cell leukemia transcription factor 1 (*PBX1*). Additional downregulated genes are *IGFBP5* and *IGFBP7*, fibronectin, *CRABP2*, *HLA-B*, the PI3 kinase p85a subunit, and select members of the Wnt signaling pathway, *Frizzled 1 and 2* (*FZD1* and *FZD2)* (Supplement 2B, available online at www.biolreprod.org).

Downregulated only at 3 h. Among the key downregulated genes that are downregulated only at 3 h (Table 3) are several zinc finger-related proteins, syndecan 1, *CCL15* (*SCYA15*), *FOXO1A*, *HOXA11*, and angiopoetin-2, as well as several members of the Wnt family, including *FZD7,* and Wnt-inducible signal pathway protein 1 (*WISP1)*. Select solute carriers are also downregulated at 3 h (Supplement 2B, available online at www.biolreprod.org).

Downregulated only at 12 h. The most highly downregulated genes at 12 h are listed in Table 3 and Supplement 2B, available online at www.biolreprod.org. One of the most highly downregulated genes is *CD24* (0.03-fold), which produces a cell surface antigen expressed on B cells and various types of carcinomas. Downregulated growth factors and related proteins include angiopoietin-1, *IGF1*, *VEGF,* and *TGFB1*. There is downregulation of structural proteins, possibly indicative of the overall tissue remodeling that takes place during the invasive phase of implantation. Some of these include collagens, *COL14A1, COL1A1, COL1A2*, and *COL3A1*. There are several Wnt family members that are downregulated, including *TCF*, *WNT4*, and *WNT5B*, as well as secreted Frizzled-related protein

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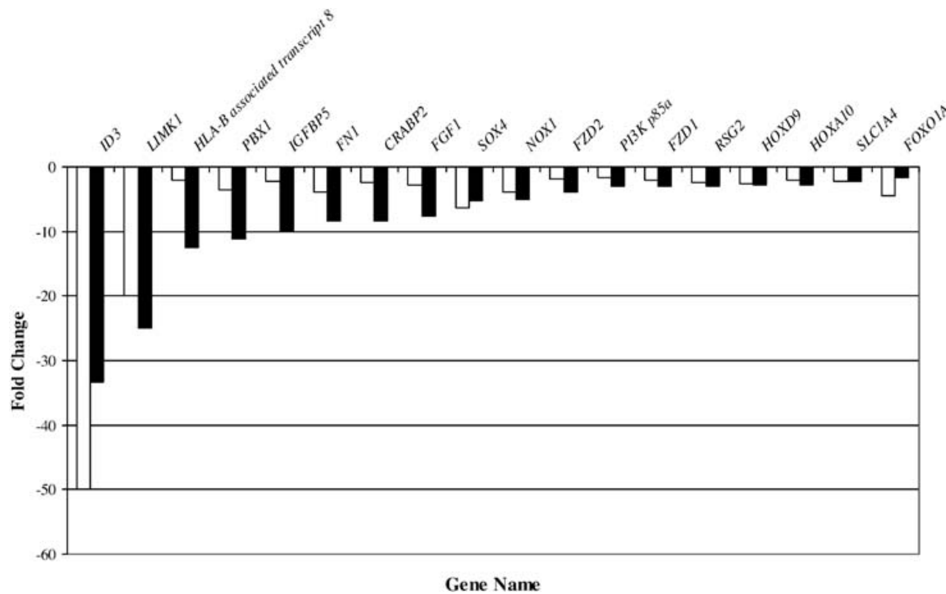

FIG. 5. Downregulated genes in decidualized human endometrial stromal cells after at 3 and 12 h of treatment with TCM, compared to controls. Gene names (abbreviations) are shown on the x-axis. The fold change is shown on the y-axis. White bars correspond to the fold change at 3 h, and black bars correspond to the fold change at 12 h.

1 (*SFRP1*). Also downregulated is *JAK1* (0.24), a member of the JAK-STAT signaling cascade, and members of the KLF factor transcription factor family. In addition, *TIMP3*, a major product of decidualized stromal cells, is downregulated in response to TCM, which is consistent with the hypothesis that the trophoblast facilitates matrix degradation as it invades the maternal deciduas (Supplement 2B, available online at www.biolreprod.org).

KEGG pathways of downregulated genes. Supplement 5B (available online at www.biolreprod.org) lists several pathways and the number of genes that are downregulated at 3 and 12 h of TCM treatment of decidualized endometrial stromal cells. The greatest numbers of genes are involved in focal adhesion, followed by genes involved in the regulation of actin cytoskeleton, ECM receptor interactions, the insulin, Wnt, and MAP kinase signaling pathways, tight junctions, cell cycle, axon guidance, and cell communication. In addition, downregulation was observed of genes involved in leukocyte transendothelial migration, ECM receptor interaction, adherens junctions, and others. Many of these pathways involve (different) genes at 3 or 12 h, likely reflecting controls on signaling.

Validation of Gene Expression

Several approaches were used to validate changes in gene expression in response to treatment of decidualized stromal cells with TCM, including real-time PCR (Fig. 6), ELISA (Fig. 7), and immunohistochemistry (Fig. 8).

Quantitative real-time PCR. For real-time PCR, nine upregulated genes and nine downregulated genes were randomly selected for validation (Table 1). Among the upregulated genes (Fig. 6A), all have increased expression by microarray analysis at both 3 and 12 h of treatment with TCM compared to controls, except for *IL8* and *CCL8*, which do not show more than a 2-fold change at 3 h. In most cases, the fold change values derived from the microarray data and the real-time PCR analysis were different in absolute magnitude, although the changes were in the same direction. This may be explained by the sensitivity of the respective assays. Of the nine genes chosen for validation, two showed statistically significant fold change after 3 h, while seven showed a statistically significant fold change after 12 h, with $P < 0.05$ (Fig. 6A).

Among the genes validated to be downregulated by quantitative RT-PCR (Fig. 6B), all have reduced expression at 12 h by microarray analysis, but only *IGFBP5* and *FGF1* also show downregulation at 3 h. For the genes chosen for validation, all, except for *IGF1*, are downregulated at both 3 and 12 h by real-time PCR (Fig. 6B). Again, this difference is likely attributed to assay sensitivity. At 3 h, only *CSRP* was statistically significantly regulated, and at 12 h, three of the nine genes demonstrated statistically significant fold changes by REST analysis.

ELISA technique. To determine whether highly regulated genes were transcribed, we investigated CXCL1 and IL8 in TCM-treated stromal cell media, compared to controls (Fig. 7). Baseline levels of CXCL1 in conditioned media at $t = 0$ were 7.05 ng/ml, which rose to 34.33 ng/ml after 3 h of treatment, compared to 14.82 ng/ml in the control condition. By 12 h of TCM treatment, the protein level increased to 118.40 ng/ml, compared to virtually no change in the control (Fig. 7A). The increase in CXCL1 was statistically significant at both 3 and 12 h of TCM treatment, compared to CCM, by paired *t*-test analysis. In addition, the CXCL1 levels were statistically significant in the TCM compared to $t = 0$ at both 3 and 12 h by paired *t*-test analysis (data not shown). However, there was no significant difference between the CCM CXCL1 levels and $t = 0$ (data not shown).

Baseline levels of IL8 at $t = 0$ were approximately 1.25 ng/ml (Fig. 7B). By 3 h of TCM treatment, they rose to 33.67 ng/ml, whereas there was little change in the control (1.73 ng/ml). By 12 h of treatment, the IL8 concentration was 61 ng/ml, compared to 2.91 ng/ml in the control. The change in IL8 levels at both 3 and 12 h of TCM treatment was significantly different from the CCM treatment, by paired *t*-test analysis. In addition, IL8 levels were significantly different at 3 and 12 h of TCM treatment, compared to $t = 0$, by paired *t*-test analysis (data not shown). There was no significant difference between the CCM and $t = 0$ by the same statistical analysis. For both CXCL1 and IL8, a repeated-measures ANOVA was used as an alternate statistical test of both time and treatment effects. In both cases, the test showed a statistically significant difference by the TCM treatment over time compared to the control, with a *P*-value that was <0.01 (data not shown).

Immunohistochemistry. While numerous genes were regulated in decidualized endometrial stromal fibroblasts over

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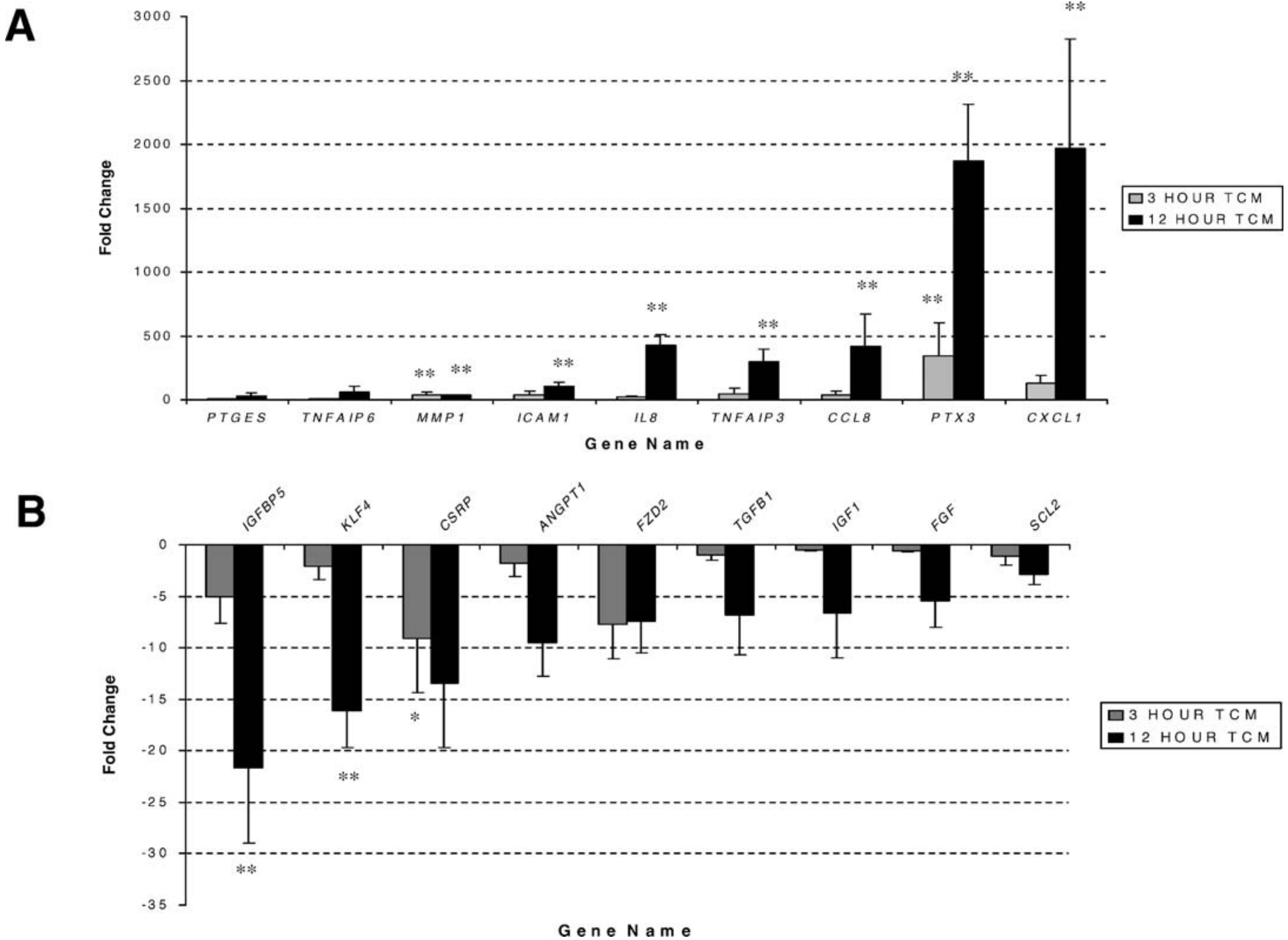

FIG. 6. Validation by real-time PCR of genes regulated in decidualized human endometrial stromal cells treated with TCM compared to controls. Real-time PCR was performed for (**A**) upregulated genes and (**B**) downregulated genes by TCM treatment compared to CCM treatment. Fold change values are plotted along with the SEM. Asterisks above each fold change value represent the results obtained by REST statistical analysis. Asterisk annotation is represented by $*P < 0.05$ and $**P < 0.01$.

the time of treatment with TCM, the question arises as to whether similar regulation occurs in vivo. To address this question, we investigated, by immunohistochemistry, select gene products in the cycling human endometrium and placental bed. Three proteins were evaluated—specifically, CXCL1, CCL8, and PTGES. Figure 8 shows that CXCL1 is highly expressed in glands but is minimally expressed in endometrial stromal fibroblasts in the cycling endometrium (panels A and B). It is absent in trophoblasts (panel C) but is highly expressed in decidual stromal cells in early pregnancy (panel D), supporting the in vitro data. CCL8 (middle row) is expressed in glands and some stroma in the cycling endometrium (panels E and F) and has minimal expression in trophoblasts and decidua (panels G and H). CCL8 was minimally regulated in decidualized endometrial stromal fibroblasts in response to TCM, consistent with these in vivo data. For PTGES (bottom row), there is abundant expression in glands in the proliferative phase (panel I) and in glands and some stromal cells in the secretory phase (panel J). While absent in placental trophoblasts and fibroblasts (panel K), PTGES is abundantly expressed in decidual fibroblasts in early pregnancy (panel L), consistent with the microarray data.

DISCUSSION

Upregulated Genes

The data reported in the present study demonstrate that decidualized human endometrial stromal cells respond to secreted products from human extravillous, invading trophoblasts by marked upregulation of genes encoding cytokines, chemokines, angiogenic factors, and others, and downregulation of genes regulating stromal mitosis and the decidual phenotype, among other processes. As the trophoblast invades the decidual stromal matrix, it recruits immune cells and eventually establishes the placental vasculature [6, 7]. On the maternal side, the decidua regulates the invasion of the trophoblast and facilitates the anchoring of the pregnancy, recruiting immune cells that promote the survival of the fetal allograft, angiogenesis, and maintaining a pathogen-free, free radical-free environment [23]. Genes that regulate most of these processes are expressed in nonconception cycles in the human endometrium during the implantation window [8, 23, 31, 41–44]. The data presented, however, suggest that the invading trophoblast secretes paracrine signals to the decidua that amplify trophoblast signals, particularly with regard to immune modulation and angiogenesis, resulting in an enriched

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cytokine milieu likely to control leukocyte influx, proliferation, and activation, as well as angiogenic and angiostatic processes in the implantation site. In addition, the data support molecular dialogs between the trophoblast and decidual stromal fibroblasts that also affect trophoblast migration and differentiation, anchoring of the placenta, and mechanisms for maintaining a sterile environment in the uterine cavity in very early pregnancy. In the present study, we focus on the immune and angiogenic phenotypes.

Immune and Angiogenic Factors

Chemokines. CXCL1 (*GRO1*), *IL6, RSAD2, CCL8, PTX3, IL8*, *PTGES*, *ICAM1*, and a variety of IFN-responsive and related genes are among the most highly upregulated genes in decidual endometrial stromal cells in response to trophoblast-secreted products (Fig. 4 and Supplement 2, available online at www.biolreprod.org). *CXCL1* and *IL8* (*CXCL8*) belong to the CXC family of chemokines that have pivotal roles in leukocyte recruitment for the control of inflammation and angiogenesis [45]. *CXCL1* is expressed in human endometrial stroma during the secretory phase of the cycle, is upregulated by in vitro decidualization, and is markedly upregulated by treating endometrial stromal fibroblasts with IL1B and TNF [46]. Red-Horse et al. [47] have demonstrated a high expression of *CXCL1* in decidual stromal fibroblasts and a high expression of *CXCR2*, its cognate receptor, in trophoblast progenitors in trophoblast column initiation sites during early gestation. These data support the trophoblast communicating to the decidua such that the latter directs cytotrophoblasts at the base of the columns to adopt the invasive phenotype. While CXCL1 is a potent chemo attractant for neutrophils, CXCR2 is minimally expressed in first-trimester decidual leukocytes [21, 47], suggesting that the CXCL1/CXCR2 pair is primarily involved in trophoblast-decidual crosstalk. The most highly expressed CXCR in decidual leukocytes is CXCR4 [47], whose primary ligand, stromal-derived factor-1, is also expressed in decidua [26, 48], although this gene was not upregulated in response to trophoblast-secreted products in the present study.

IL8 is among the most highly upregulated, early-response genes in endometrial stromal cells exposed to trophoblast-secreted products. In the cycling human endometrium, IL8 is highly expressed in the perivascular stroma, with the most intense immunostaining observed during the premenstrual phase and minimal expression during the rest of the cycle [8, 49–51]. However, it is abundantly expressed in pregnancy decidua [8]. IL8 is a chemokine that is a major mediator of the inflammatory response and is a chemo attractant for neutrophils [52] and T lymphocytes [53]. It is highly upregulated by IL1, TNF, and thrombin treatment of cultured decidual stromal fibroblasts [54], and its cognate receptor, CXCR1, is expressed in first-trimester decidual leukocytes [47]. Control of leukocyte trafficking into the implantation site involves the combined actions of chemokines and adhesion molecules, with IL8 and ICAM1 featured prominently in this process [55]. It is of interest that the decidualized endometrial stromal cell, in response to the invading trophoblast, exhibits coordinated upregulation of *IL8* and *ICAM1*, suggesting a role for the decidual fibroblast in intrastromal leukocyte trafficking and perhaps activation. These data are supported by the earlier findings of Chen et al. [56] on genes enriched in first-trimester decidua, compared to chorionic villi, in which there was upregulation of *ICAM1* as well as IFN regulatory factor 7, *IL1RL1*, superoxide dismutase, and several other genes observed in the present study. Interesting, however, is that many genes were not enriched, suggesting that paracrine

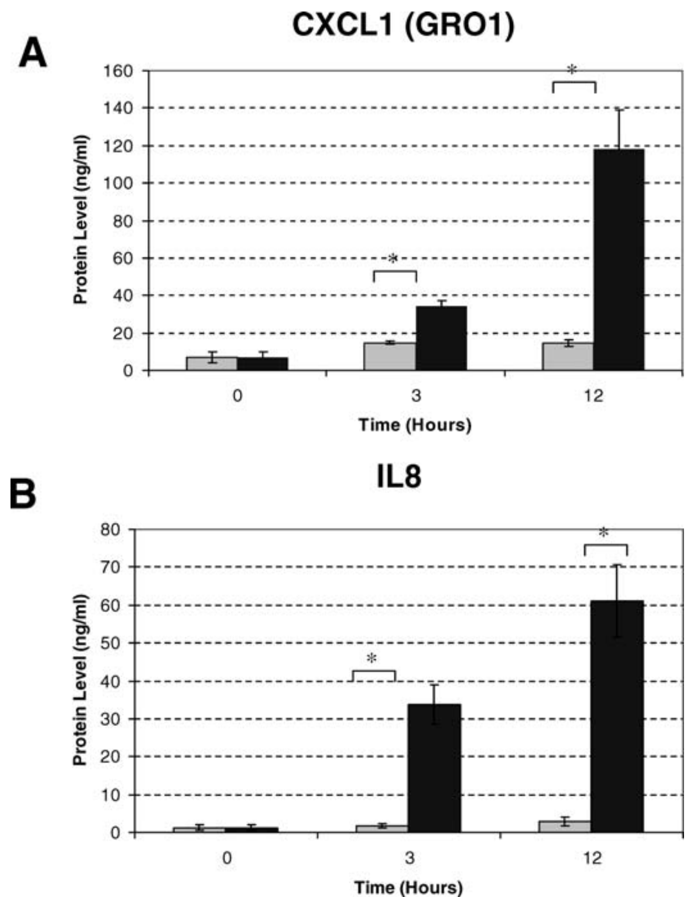

FIG. 7. Validation by ELISA of CXCL1 and IL8 protein expression. ELISA data on CXCL1 (**A**) and IL8 (**B**) showing the increasing amounts of each protein with time of TCM treatment. Hatched bars represent values in response to CCM, and solid black bars represent levels in response to TCM. Asterisks indicate results of paired *t*-test analysis. Asterisk annotation is represented by $*P < 0.05$.

interactions in microenvironments are very important in the placental bed and early implantation.

CXC chemokines that contain the ELR motif (e.g., IL8 and CXCL1) are also potent promoters of angiogenesis and mediate their angiogenic activity by binding and activating CXCR2 on the endothelium [45]. IL8 is a potent angiogenic factor, even in the absence of preceding inflammation [45, 57], and interestingly, in the process of wound healing, CXCL1 and IL8 cooperatively support neutrophil migration to the superficial wound bed [45]. That both chemokines are coexpressed in endometrial stromal cells in response to trophoblast-secreted products is consistent with their cooperative actions in the placental bed. The data in the present study suggest that the rapid upregulation of *IL8* (and *CXCL1*) is mediated through a direct action of the trophoblast-secreted product(s) on the decidualized endometrial stromal cell, rather than an induction through other factors.

TNFAIP6 *gene.* The cytokine-inducible protein encoded by this gene is a secretory protein that contains a hyaluronan-binding domain that is involved in ECM stability and cell migration. It specifically binds to hyaluronan and forms a stable complex with the inter-alpha-inhibitor, which is important in the protease network associated with inflammation. Another highly upregulated gene, *PTX3*, does not bind directly to hyaluronan, but binds to TNFAIP6, enabling the cross-linking of hyaluronan chains as multimolecular com-

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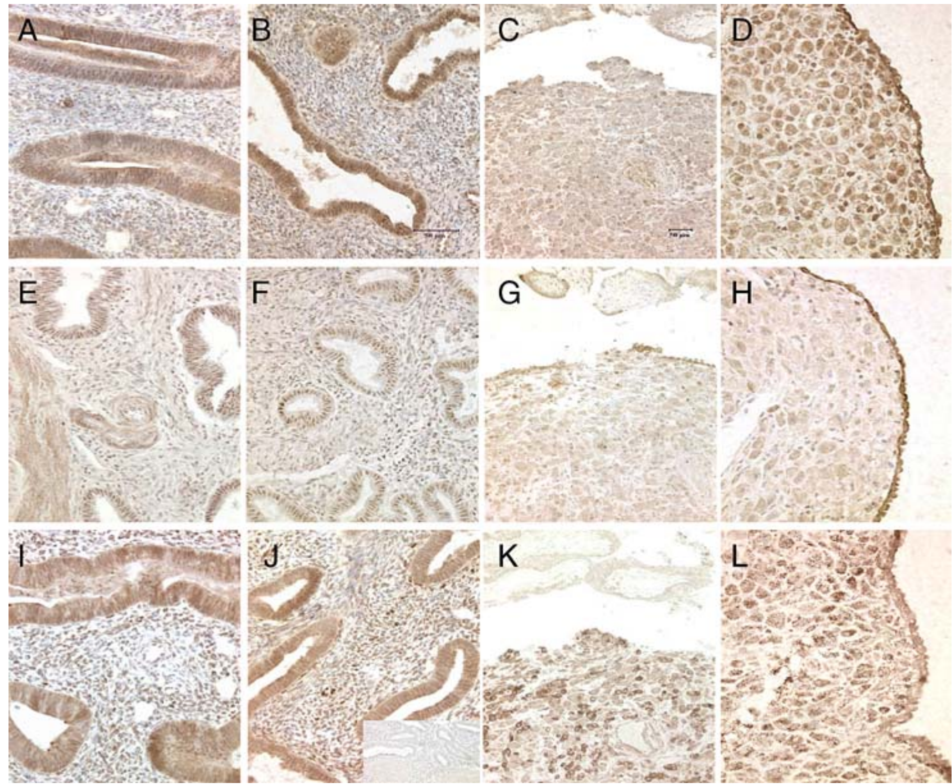

FIG. 8. Immunohistochemical localization of select gene products in the cycling human endometrium and placental bed. Top row, CXCL1: panel **A**, proliferative-phase endometrium; panel **B**, secretory-phase endometrium; panel **C**, implantation site with placenta and decidua present; panel **D**, decidua. Middle row, CCL8: panel **E**, proliferative-phase endometrium; panel **F**, secretory-phase endometrium; panel **G**, implantation site with placenta and decidua present; panel **H**, decidua. Bottom row, PTGES: panel **I**, proliferative-phase endometrium; panel **J**, secretory-phase endometrium (inset, preimmune control); panel **K**, implantation site with placenta and decidua present; panel **L**, decidua. Original magnification **A, B, D–F, H–J,** and **L** ×40 and **C, G,** and **K** ×20.

plexes. The expression of *TNFAIP6* can be induced by TNF, IL1, and mechanical stimuli in vascular smooth muscle cells that lead to proteoglycan synthesis and aggregation. Modulation of the proteolytic network associated with inflammatory processes may be a mechanism whereby TNFAIP6 inhibits inflammation. The activation of the *TNFAIP6* gene by proinflammatory cytokines and the presence of TNFAIP6 protein in inflammatory lesions suggest a role in the negative feedback control of the inflammatory response [58, 59]. Both PTX3 and TNFAIP6 are synthesized in the ovary prior to ovulation, and both become components of an expanding viscoelastic cumulus matrix surrounding the oocyte before it is released from the follicle at the ovarian surface. Since this gene is markedly upregulated in decidualized endometrial stromal cells after 3 and 12 h of exposure to trophoblast-secreted products, it is possible that it is involved in anchoring the conceptus in the stromal matrix and also in enabling the invasive trophoblast to migrate through the stroma.

IFN-regulated and -related genes. Effects of trophoblast-secreted products on decidualized human endometrial fibroblasts result in the upregulation of numerous IFN-regulated and -related genes. These observations are strikingly similar to events occurring in the endometrium during early pregnancy in ruminants. For example, IFNτ regulates the expression of specific genes in the endometrial stroma (and deep glandular epithelium) of the ewe, including *STAT1*, *STAT2* [60, 61], MHC class I and β2 microglobulin [62], IFN regulatory factor 1 (*IRF1*) and *IRF9* [60], Mx family members [63], *OAS*, and *ISG15* [64]. These are some of the most highly regulated genes in human endometrial stromal cells in response to human TCM (Fig. 4B), suggesting that there is an IFN-like response in the initial stages of the trophoblast-decidual stromal interactions. Importantly, despite extensive efforts, IFN and similar viral activity could not be confirmed in human trophoblasts or TCM (unpublished results). Furthermore, we did not find any upregulation of IFN genes (α, β, or γ) in decidualized stromal cells exposed to TCM, although many genes that are upregulated and affect signaling pathways in the stromal response to trophoblast-secreted products appear to involve an IFN-like response. Some IFN-regulated genes have also been observed in the peri-implantation period in mice [9, 65], and while an IFNτ-like ligand has not been found in human trophoblasts or other cells in the placental bed, the endometrium is known to be responsive to IFNγ, presumably secreted by uterine NK cells in the stroma. For example, IFNγ antagonizes cAMP-mediated prolactin expression by decidualizing human endometrial stromal fibroblasts, an effect that is mediated by *STAT1* upregulation, phosphorylation, and translocation to the nucleus [66]. This involves a protein inhibitor of activated STAT that complexes with the P_4 receptor in human endometrial stromal cells [67]. IFNγ is believed to initiate uterine vascular modification and promote decidual cell survival in a mouse model [68], and it induces apoptosis in cytotrophoblasts and syncytiotrophoblasts [69]. In our experimental system, there was no evidence of trophoblast apoptosis, and the molecular entities that elicit IFNγ and IFNτ responses remain to be elucidated.

IL6 *gene synthesis*. *IL6* is synthesized by mononuclear phagocytes, vascular endothelial cells, fibroblasts, and other cells, in response to IL1 and TNF [38]. Its receptor, IL6Ra, dimerizes with the shared gp130 signal-transducing subunit, and ligand binding results in the activation of the JAK-STAT signaling pathway. IL6 is a multifunctional cytokine, and among its multiple actions, it stimulates the production of neutrophils from bone marrow and stimulates the growth of antibody-producing differentiated B lymphocytes. Its role in decidua is unknown, although similar functions may protect this tissue against infection when trophoblast cells are present. Interestingly, the OSMR (IL6) is also upregulated in stromal cells exposed to trophoblast products, as is an inhibitor of its signaling, *SOCS3* (suppressor of cytokine signaling) (Supplement 2A, available online at www.biolreprod.org).

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Signaling Pathways

It is not unanticipated that cytokine/chemokine signaling components are among the most highly upregulated genes in decidualized stromal cells in response to trophoblast products, given that the immune response is one of the most highly represented GO categories of upregulated genes at 3 and 12 h of treatment. There are several JAK-STAT pathways involved in responses to cytokines [38]. For example, IFNα, IFNβ, and IFNγ signal through STAT1 and STAT2, both of which were highly upregulated in stromal cells in response to trophoblast-secreted products. IFNα, IFNβ, and IFNγ and cytokines with gp130 (e.g., IL6) signal through JAK1 (another gene highly upregulated in stromal cells), while IFNγ also uses JAK2. Inhibition of JAK-STAT pathways is effected by a family of SOCSs, which are generally induced by the same cytokine that activates the pathway [38]. Relevant to activation of the stromal cell in response to trophoblast-secreted products is the upregulation of *SOCS3*, an inhibitor of IL6 signaling. Interestingly, *SOCS1*, an inhibitor of IFNγ signaling, is not regulated in stromal cells in response to trophoblast-secreted products. Genes for select members of MAP kinase signaling pathways were also found to be upregulated in the present study. While the precise activators of these pathways are uncertain (see below), several ligands may participate, including IGF2, which is a major product of the invading trophoblast [70].

Paracrine Signals

Of major interest is the identity of the products in TCM that elicit the responses in the decidualized stromal cells, observed in the present study. Given the signaling pathways and regulated genes in endometrial stromal cells in response to trophoblast-secreted products, several candidates emerge—primarily IL1, TNF, and member(s) of the IFN family, as well as IGF2 and perhaps hCG and P_4. It is known that IL1B is expressed in human extravillous trophoblasts, macrophages, and decidual stromal fibroblasts [71, 72] and that purified cytotrophoblasts secrete IL1B in vitro in correspondence with their invasive potential [73]. In addition, IL1B stimulates proMMP-3 expression in primate endometrial stromal fibroblasts acting through the MAP kinase pathway [74, 75]. Several genes that were upregulated in the present study are induced by IL1B and TNF, including *PLAU, PTX3, PTGES, CXCL1, IL8, TNFAIPs, IL6*, and *MMP3*. These are not upregulated by P_4 treatment of stromal cells [25, 26] (unpublished results). Binding of TNF to its receptor stimulates gene expression (and inhibits apoptosis) primarily through activation of transcription factors, including NF-κB (upregulated in this study) [38]. Many of the effects of IL1 are similar to those of TNF because IL1 signaling shares members with the TNF signaling pathway. TNF also stimulates IL1 in many cell types, thereby enhancing its own effects [38]. TNF stimulates cytokines in endothelial cells and macrophages that increase the affinity of leukocyte integrins for their ligands and also stimulate leukocyte chemo taxis and recruitment. IL1 has similar effects, and if one or both of these ligands are involved in the communication between the trophoblast and the decidual fibroblast, then the latter may acquire a phenotype of attracting leukocytes (and perhaps the trophoblast) and facilitating their migration into the implantation site. Overall, the response of the stromal fibroblast to trophoblast-secreted products is one of a proinflammatory response, highly suggestive of IL1 actions, TNF actions, or both. In a recent study from our laboratory, IL1B upregulated *CXCL1* and *IL8* mRNA in decidualized endometrial stromal cells, consistent with observations by Nasu et al. [46], whereas IFNγ and ovine IFNτ were without effect (unpublished results).

Striking, also, is the number of IFN-regulated and -related genes in decidualized endometrial stromal cells in response to trophoblast-secreted products. As discussed above, the ligand promoting this response is uncertain at this time, although the signaling components through the JAK-STAT pathway and IFN-induced genes suggest a member of the IFNα, IFNβ, or IFNγ family. Intriguing is that a molecule similar to IFNτ may be involved, and this is currently under investigation in our laboratory. IGF2 is highly expressed in vivo in invading trophoblasts at the leading front in the process of implantation in humans [70]. It is of interest that several genes upregulated in decidualized endometrial stromal fibroblasts observed in the present study are similarly regulated by IGF2 (e.g., *IL8*, *CCL8,* IFNα/β receptor, *TNFAIP2, TNFAIP3, TNFAIP6*, *ICAM1*, metallothioneins [unpublished results]). Thus, it is possible that IGF2 is among the trophoblast-secreted products that affect decidual stromal cell function. Also, a major product of the trophoblast is hCG. It is likewise possible that some of the observed effects are due to this gonadotropin, and this awaits further investigation.

The present study has used an in vitro model to mimic events occurring in early pregnancy. While both the stromal cell cultures and the trophoblast cultures were highly pure (>99%) and are devoid of leukocytes and viral activities (see above), the possibility exists that cells other than stromal fibroblasts are present in the cultures. However, the in vivo validation suggests that minor contaminants, if present, do not contribute significantly to the gene expression profiles observed. Stromal cells may need the in vivo ECM for appropriate functioning, although they synthesize ECM in a way that is similar to that found in vivo in response to the decidualization stimulus. In addition, cells in vitro may behave very differently from cells in vivo, although the limited immunohistochemical study performed suggests that our observations are not unique to in vitro conditions. The present study has demonstrated that trophoblast-secreted products, yet to be identified, have profound effects on human decidualized endometrial stromal fibroblasts, the cell type encountered during the invasive phase of implantation as the invading trophoblast journeys through the maternal decidua. These effects involve a network of chemokine ligand/receptors, angiogenic/static factors, and other factors at the decidual-placental interface, likely of importance in trophoblast migration and differentiation, as well as leukocyte trafficking, migration, and activation. On the basis of the results reported in the present study, decidualized stromal fibroblasts in close proximity to invading trophoblasts have characteristics of immune cells that orchestrate the enrichment of cytokines and chemokines in the implantation site. These cells likely differ considerably from the decidual stromal cells in other parts of the endometrium during early pregnancy. While the players involved at the trophoblast-decidual interface await further definition, the responses observed in the present study underscore the complexity of these interactions and some redundancy of the functions likely to optimize the cytokine-chemokine-angiogenic milieu in the invasive phase of implantation in human pregnancy.

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