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Original articles – Obstetrics

Characterization of the myometrial transcriptome and biological pathways of spontaneous human labor at term

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Abstract

Aims: To characterize the transcriptome of human myometrium during spontaneous labor at term.
Methods: Myometrium was obtained from women with (n = 19) and without labor (n = 20). Illumina® HumanHT-12 microarrays were utilized. Moderated *t*-tests and false discovery rate adjustment of P-values were applied. Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) was performed for a select set of differentially expressed genes in a separate set of samples. Enzyme-linked immunosorbent assay and Western blot were utilized to confirm differential protein production in a third sample set.
Results: 1) Four hundred and seventy-one genes were differentially expressed; 2) gene ontology analysis indicated enrichment of 103 biological processes and 18 molecular functions including: a) inflammatory response; b) cytokine activity; and c) chemokine activity; 3) systems biology pathway analysis using signaling pathway impact analysis indicated six significant pathways: a) cytokine-cytokine receptor interaction; b) Jak-STAT signaling; and c) complement and coagulation cascades; d) NOD-like receptor signaling pathway; e) systemic lupus erythematosus; and f) chemokine signaling pathway; 4) qRT-PCR confirmed over-expression of prostaglandin-endoperoxide synthase-2, heparin binding epidermal growth factor (EGF)-like growth factor, chemokine C-C motif ligand 2 (CCL2/MCP1), leukocyte immunoglobulin-like receptor, subfamily A member 5, interleukin (IL)-8, IL-6, chemokine C-X-C motif ligand 6 (CXCL6/GCP2), nuclear factor of kappa light chain gene enhancer in B-cells inhibitor zeta, suppressor of cytokine signaling 3 (SOCS3) and decreased expression of FK506 binding-protein 5 and aldehyde dehydrogenase in labor; 5) IL-6, CXCL6, CCL2 and SOCS3 protein expression was significantly higher in the term labor group compared to the term not in labor group.
Conclusions: Myometrium of women in spontaneous labor at term is characterized by a stereotypic gene expression pattern consistent with over-expression of the inflammatory response and leukocyte chemotaxis. Differential gene expression identified with microarray was confirmed with qRT-PCR using an independent set of samples. This study represents an unbiased description of the biological processes involved in spontaneous labor at term based on transcriptomics.

Keywords: Aldehyde dehydrogenase (ALDH2); chemokine C-C motif ligand 2 (CCL2/MCP-1); chemokine C-X-C motif ligand 6 (CXCL6/GCP2); FK506 binding-protein 5 (FKBP5); heparin binding EGF-like growth factor (HBEGF); inflammasome; inflammation; interleukin (IL)-6, -8; leukocyte immunoglobulin-like receptor, subfamily A, member 5 (LILRA5/LIR9); microarray; nuclear factor of kappa light chain gene enhancer in B-cells inhibitor zeta (NFKBIZ); parturition; pregnancy; progesterone; prostaglandin-endoperoxide synthase-2 (PTGS2/COX2); suppressor of cytokine signaling 3 (SOCS3); systems biology.

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Introduction

Parturition is a complex process involving myometrial activation, cervical ripening, and membrane/decidual activation (the common pathway of labor) [2, 21, 30, 34–37, 41, 114,

149, 169–171, 172, 210, 212, 217, 220, 236, 246, 247, 298, 313, 327, 333]. Evidence suggests that in preparation for labor, the myometrium attains an increasingly contractile phenotype [27, 29, 53, 55, 59, 62, 66, 68, 100, 103, 106, 108, 110, 112, 113, 122–124, 146, 174, 176, 179, 181, 183, 188, 190, 196, 216, 225, 280–282, 296, 310, 315, 321], while the cervix undergoes preparatory changes including cervical ripening and dilatation [61, 68, 112, 113, 132, 134, 143, 147, 151, 177, 184, 185, 218, 219, 225, 272, 275, 276, 282, 296, 297, 311, 312, 321, 330, 337]. Labor disorders such as preterm labor and abnormal parturition at term represent abnormalities of one or more components of the common pathway of parturition and are associated with increased morbidity and mortality [101, 102, 117, 118, 121, 148]. The application of high-dimensional biology techniques holds promise to assist in the understanding of parturition (term and preterm). While the process of labor and delivery is vital to the survival of most viviparous species, its physiology and pathology in humans remains to be elucidated.

High-dimensional biology techniques (genomics, transcriptomics, proteomics, etc.) provide the means by which comprehensive and unbiased insight into physiologic events, such as parturition, can be established [142, 158, 163, 244, 253, 263, 304]. Previous investigators have discovered differential gene and/or protein expression in the chorioamniotic membranes [128], amniotic fluid [23, 24, 43, 50, 125, 197, 222, 224, 248, 270, 322], umbilical cord blood [182], uterine cervix [132–135, 143, 205, 234, 324], and human myometrium [1, 13, 25, 39, 90, 91, 136, 214] in preterm and term labor (TL). However, the biological processes, molecular functions, and pathways associated with spontaneous term parturition have not been described and the regulatory mechanisms remain poorly understood. We undertook this study in order to characterize the transcriptome of human myometrium during normal labor at term to gain understanding of global changes in gene expression using an unbiased approach.

Materials and methods

A prospective study was performed in which myometrium was obtained from women undergoing cesarean section at term ($\geq$37 weeks) in the following groups: 1) not in labor (n = 20); and 2) spontaneous labor (n = 19). (Please see supplementary material for details.)

Eligible patients were enrolled at Hutzel Women's Hospital (Detroit, MI, USA). All women provided written informed consent prior to the collection of myometrial samples. The collection and utilization of the samples for research purposes was approved by the Institutional Review Board of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD/NIH/DHHS, Bethesda, MO, USA), and the Human Investigation Committee of Wayne State University (Detroit, MI, USA).

Sample collection

Myometrial tissue samples were collected from the hysterotomy site in the lower uterine segment during cesarean section following delivery of the placenta from the midpoint of the superior aspect of the uterine incision using Metzenbaum scissors and measured approximately 1.0 cm^3. Tissues were snap-frozen in liquid nitrogen, and were kept at −80°C until use.

RNA isolation

Total RNA was isolated from snap-frozen myometrium using TRI Reagent® combined with the Qiagen RNeasy Lipid Tissue kit protocol (Qiagen, Valencia, CA, USA) according to the manufacturers' recommendation. The RNA concentrations and the A260 nm/A280 nm ratio were assessed using a NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA). RNA integrity numbers were determined using the Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA).

Microarray analysis and real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

The Illumina® HumanHT-12 v3 expression microarray platform (Illumina, San Diego, CA, USA) was used to assess the expression levels in each individual specimen following the manufacturer's instructions. qRT-PCR assays were performed for selected genes on an independent set of myometrium samples [term with (n = 10) and without (n = 10) labor] to determine whether the microarray results could be confirmed. The Biomark™ System (Fluidigm, San Francisco, CA, USA) was utilized to perform high-throughput qRT-PCR confirmation. (Please see supplementary material for details.)

Enzyme-linked immunosorbent assay and immunoblot

A third independent set of myometrial samples were obtained from women at term with (n = 9) and without labor (n = 11). Myometrial concentrations of interleukin (IL)-6, chemokine C-C motif ligand 2 (CCL2), and IL-8 were determined with specific enzyme-linked immunoassays (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's instructions. Protein expression of suppressor of cytokine signaling 3 (SOCS3) and CXCL6 was analyzed using immunoblot. (Please see supplementary material for details.)

Statistical analysis

Demographic and clinical characteristics of the study groups were compared using the Pearson's χ^2-test and the Fisher's exact test for proportions and Kruskal-Wallis and the Mann-Whitney *U*-test for continuous variables. SPSS v.12 (SPSS Inc., Chicago, IL, USA) was used. A $P < 0.05$ (two-tailed) was considered significant.

Microarrays, qRT-PCR, and ELISA

A moderated *t*-test was applied to test differential expression, and a false discovery rate (FDR) adjustment of the P-value was performed to correct for multiple testing. Genes with an FDR < 0.05 and a fold change > 1.5 were considered significant. Gene ontology analysis was performed using an over-representation approach implemented in the Onto-Express [156, 157] site and GOstats [95] software packages. Pathway analysis was performed on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database using an enrichment analysis as well as the Signaling Pathway Impact Analysis (SPIA) [69, 303]. SPIA is based on a systems biology approach [69] and takes into account gene-gene signaling interactions as well as the magnitude and direction of gene expression changes to determine significantly impacted pathways. The

MetaCore database (Gene Go, Inc., St. Joseph, MI, USA) was also mined. (Please see supplementary material for details.)

Analysis for qRT-PCR was performed using an equal variance two-sample one-tailed *t*-test. Protein concentrations determined by enzyme-linked immunosorbent assay (ELISA) were analyzed using one-tailed Mann-Whitney *U*-tests. One-tailed tests were used because there was a prior hypothesis about the direction of change based upon microarray results.

Results

Table 1 displays the demographic and clinical characteristics of each study group. There were no differences among the groups.

Microarray results

Discriminant analysis demonstrated significant changes in the transcriptome of human myometrium between women with and without labor. In total, 538 probes corresponding to 471 unique genes were differentially expressed. The top 50 differentially expressed probes overexpressed and underexpressed in spontaneous TL are listed in Tables 2 and 3, respectively. The results of microarray profiling are depicted in Figure 1. The volcano plot (Figure 1A) shows the magnitude and significance of differential gene expression. Principal component analysis (PCA) [302] (Figure 1B) was performed to reduce the dimensionality of the microarray data from that of all genes tested to three-dimensions. The heat map in Figure 1C uses a color scale to show the consistency of the expression levels within each group of samples as well as the differences between the groups that led to positive test results.

In order to gain further insight into the biology of the differential gene expression, Gene Ontology enrichment analysis was employed. A total of 103 biological processes were associated with spontaneous TL. The most significantly enriched biological processes included: 1) inflammatory response; 2) response to wounding; and 3) response to external stimulus. Eighteen molecular functions were enriched in spontaneous TL including: 1) cytokine activity; 2) heparin binding; 3) receptor binding; 4) chemokine activity; and 5) chemokine receptor binding (Table 4A).

Pathway analysis

Pathway analysis on the KEGG database indicated significant enrichment of four pathways: 1) cytokine-cytokine receptor interaction; 2) Jak-STAT signaling pathway; 3) complement and coagulation cascade; and 4) ascorbate and aldarate metabolism. Based on over-representation analysis, 22 pathways in the MetaCore database were significant including IL-17 signaling. Of interest, 19 of 22 (86%) enriched pathways were involved in the biology of inflammation. The MetaCore map of differentially expressed genes in the IL-17 signaling pathway is depicted in Figure 2. Although gene regulation network models may be derived from experimental data spanning multiple systems and not be completely applicable to a single system, an entire net-

Table 1 Demographic and clinical characteristics of the study groups.

Category	Term not in labor Microarray (n = 20)	Term labor Microarray (n = 19)	Term not in labor qRT-PCR (n = 10)	Term labor qRT-PCR (n = 10)	Term not in labor Immunoblot/ELISA (n = 11)	Term labor Immunoblot/ELISA (n = 9)	P-value
Maternal age (years)	33 (21–39)	27 (19–39)	27 (19–33)	26 (20–40)	24 (19–30)	27 (21–38)	NS
African-American ethnicity	75 (15/20)	70 (14/20)	80 (8/10)	90 (9/10)	80 (8/10)	80 (8/10)	NS
BMI (kg/m^2)	28 (18.2–47.2)	28.7 (22.1–54.6)	31.8 (21.7–61)	24.2 (21–31.3)	29.6 (22.3–38.4)	27.7 (21–33.4)	NS
Parity	1 (0–4)	0 (0–5)	1 (0–6)	1 (0–5)	1 (0–4)	1 (0–5)	NS
Gestational age at delivery (weeks)	38.7 (37–41.9)	39.3 (37–41.3)	39.1 (38.9–41.3)	38.9 (37–41)	39.1 (38.7–41)	39.2 (38.9–40.5)	NS
Birthweight (g)	3070 (2545–3805)	3150 (2570–3740)	3330 (3090–3930)	3245 (2645–3740)	3490 (3010–3980)	3280 (2870–3840)	NS

Values are expressed as percentage (number) or median (range).
qRT-PCR = real-time quantitative reverse transcriptase-polymerase chain reaction, ELISA = enzyme linked-immunosorbent assay, NS = not significant, BMI = body mass index.

Table 2 Microarray results. Top 50 probes with overexpression in human myometrium during spontaneous term labor.

ENTREZ gene ID	Symbol	Gene name	Fold change	FDR corrected P-value
8507	ENC1	Ectodermal-neural cortex	1.70	0.0002
10630	PDPN	Podoplanin	2.09	0.0007
8703	B4GALT3	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3	1.59	0.0008
3872	KRT17	Keratin 17	4.22	0.0008
10797	MTHFD2	Methylenetetrahydrofolate dehydrogenase 2	1.87	0.0010
58477	SRPRB	Signal recognition particle receptor, B subunit	1.52	0.0010
353514	LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A member 5	3.48	0.0010
22936	ELL2	Elongation factor, RNA polymerase II, 2	1.85	0.0010
165	AEBP1	AE binding protein 1	1.98	0.0010
64651	AXUD1	AXIN1 up-regulated 1	2.57	0.0012
117247	SLC16A10	Solute carrier family 16, member 10	2.45	0.0012
6280	S100A9	S100 calcium binding protein A9	3.52	0.0012
9941	EXOG	Endo/exonuclease (5′–3′), endonuclease G-like	3.90	0.0013
4489	MT1A	Metallothionein 1A	3.56	0.0015
366	AQP9	Aquaporin 9	3.55	0.0016
4502	MT2A	Metallothionein 2A	3.41	0.0018
732360	LOC732360	Similar to G/T mismatch-specific thymine DNA glycosylase; pseudogene	1.53	0.0019
6279	S100A8	S100 calcium binding protein A8	3.85	0.0020
10221	TRIB1	Tribbles homolog 1 (*Drosophila*)	2.56	0.0020
26585	GREM1	Gremlin 1, cysteine knot superfamily, homolog (*Xenopus laevis*)	3.22	0.0021
5142	PDE4B	Phosphodiesterase 4B, cAMP-specific	2.27	0.0021
10135	NAMPT	Nicotinamide phosphoribosyltransferase	2.68	0.0023
5744	PTHLH	Parathyroid hormone-like hormone	1.52	0.0023
1647	GADD45A	Growth arrest and DNA-damage-inducible, alpha	2.06	0.0023
8140	SLC7A5	Solute carrier family 7 member 5	2.74	0.0024
4837	NNMT	Nicotinamide N-methyltransferase	1.87	0.0025
3576	IL-8	Interleukin-8	10.36	0.0025
90007	MIDN	Midnolin	1.70	0.0025
23560	GTPBP4	GTP binding protein 4	1.51	0.0025
1735	DIO3	Deiodinase, iodothyronine, type III	2.52	0.0025
85450	ITPRIP	Inositol 1,4,5-triphosphate receptor interacting protein	2.15	0.0025
25819	CCRN4L	CCR4 carbon catabolite repression 4-like (*S. cerevisiae*)	1.90	0.0025
29950	SERTAD1	SERTA domain containing 1	1.62	0.0025
5743	PTGS2	Prostaglandin-endoperoxide synthase 2	3.86	0.0025
4501	MT1X	Metallothionein 1X	2.82	0.0026
22856	CHSY1	Chondroitin sulfate synthase 1	1.79	0.0026
23764	MAFF	V-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	1.99	0.0026
1847	DUSP5	Dual specificity phosphatase 5	2.13	0.0026
9446	GSTO1	Glutathione S-transferase omega 1	1.57	0.0026
26585	GREM1	Gremlin 1, cysteine knot superfamily, homolog (*Xenopus laevis*)	1.63	0.0028
57761	TRIB3	Tribbles homolog 3 (*Drosophila*)	1.55	0.0028
5209	PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1.60	0.0028
3099	HK2	Hexokinase 2	1.64	0.0029
4084	MXD1	MAX dimerization protein 1	1.99	0.0029
57647	DHX37	DEAH (Asp-Glu-Ala-His) box polypeptide 37	1.58	0.0031
5292	PIM1	Pim-1 oncogene	2.35	0.0031
3566	IL-4R	Interleukin-4 receptor	1.83	0.0031
51129	ANGPTL4	Angiopoietin-like 4	3.04	0.0032
2357	FPR1	Formyl peptide receptor 1	3.02	0.0032
199675	C19orf59	Chromosome 19 open reading frame 59 (mast cell-expressed membrane protein 1)	4.10	0.0032

FDR = false discovery rate.

work module in the lower right quadrant of Figure 2, containing I-κB, NF-κB, and several NF-κB targets, is upregulated in spontaneous TL. This result, for the first time, presents a completely experimentally validated gene sub-network from myometrial transcriptome data.

While four significant pathways were identified by simple pathway enrichment methods, the systems biology-based pathway analysis implemented in SPIA yielded a total of six significant pathways: 1) cytokine-cytokine receptor interaction ($P < 0.001$); 2) complement and coagulation cascade

Table 3 Microarray results. Top 50 probes with underexpression in human myometrium during spontaneous term labor.

ENTREZ gene ID	Symbol	Gene name	Fold change	FDR corrected P-value
1580	CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	–2.73	4.12 E-06
1580	CYP4B1	Cytochrome P450, family 4, subfamily B, polypeptide 1	–2.24	0.00005
55342	STRBP	Spermatid perinuclear RNA binding protein	–1.62	0.00006
3207	HOXA11	Homeobox A11	–1.81	0.00011
126823	KLHDC9	Kelch domain containing 9	–1.66	0.00012
1728	NQO1	NAD(P)H dehydrogenase, quinone 1	–2.13	0.00016
7743	ZNF189	Zinc finger protein 189	–1.85	0.00016
126823	KLHDC9	Kelch domain containing 9	–1.90	0.00019
5641	LGMN	Legumain	–1.64	0.00023
85004	RERG	RAS-like, estrogen-regulated, growth inhibitor	–2.52	0.00030
145781	GCOM1	GRINL1A complex locus	–1.72	0.00033
2289	FKBP5	FK506 binding protein 5	–1.59	0.00042
65009	NDRG4	NDRG family member 4	–1.75	0.00060
100132684	LOC100132684	Hypothetical protein LOC100132684 (C14ORF132)	–2.09	0.00062
6001	RGS10	Regulator of G-protein signaling 10	–1.50	0.00062
2861	GPR37	G protein-coupled receptor 37	–2.20	0.00062
25959	KANK2	KN motif and ankyrin repeat domains 2	–1.82	0.00062
51284	TLR7	Toll-like receptor 7	–1.69	0.00062
5733	PTGER3	Prostaglandin E receptor 3	–2.24	0.00062
284	ANGPT1	Angiopoietin 1	–1.75	0.00069
284	ANGPT1	Angiopoietin 1	–2.22	0.00071
3489	IGFBP6	Insulin-like growth factor binding protein 6	–1.62	0.00080
8718	TNFRSF25	Tumor necrosis factor receptor superfamily, member 25	–1.54	0.00084
5334	PLCL1	Phospholipase C-like 1	–2.57	0.00084
2857	GPR34	G protein-coupled receptor 34	–1.84	0.00084
6414	SEPP1	Selenoprotein P, plasma, 1	–2.46	0.00096
256691	MAMDC2	MAM domain containing 2	–3.30	0.00096
3757	KCNH2	Potassium voltage-gated channel, subfamily H, member 2	–2.29	0.00096
145781	GCOM1	GRINL1A complex locus	–1.58	0.00097
27175	TUBG2	Tubulin, gamma 2	–1.74	0.00097
51435	SCARA3	Scavenger receptor class A, member 3	–1.64	0.00115
6035	RNASE1	Ribonuclease, RNase A family, 1	–2.26	0.00123
6035	RNASE1	Ribonuclease, RNase A family, 1	–2.08	0.00125
6543	SLC8A2	Solute carrier family 8, member 2	–1.95	0.00125
130814	PQLC3	PQ loop repeat containing 3	–1.70	0.00133
10608	MXD4	MAX dimerization protein 4	–1.70	0.00143
4286	MITF	Microphthalmia-associated transcription factor	–1.54	0.00154
3033	HADH	Hydroxyacyl-coenzyme A dehydrogenase	–2.13	0.00154
5733	PTGER3	Prostaglandin E receptor 3	–1.84	0.00160
4056	LTC4S	Leukotriene C4 synthase	–1.78	0.00160
79921	TCEAL4	Transcription elongation factor A (SII)-like 4	–1.71	0.00177
2018	EMX2	Empty spiracles homeobox 2	–1.58	0.00181
463	ZFHX3	Zinc finger homeobox 3	–1.52	0.00188
10826	C5orf4	Chromosome 5 open reading frame 4	–2.28	0.00190
9459	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor 6	–1.60	0.00201
9068	ANGPTL1	Angiopoietin-like 1	–1.59	0.00204
79148	MMP28	Matrix metallopeptidase 28	–1.67	0.00207
10742	RAI2	Retinoic acid induced 2	–1.55	0.00222
54510	PCDH18	Protocadherin 18	–1.78	0.00230
7106	TSPAN4	Tetraspanin 4	–1.52	0.002339

FDR = false discovery rate.

($P < 0.001$); 3) Jak-STAT signaling ($P < 0.001$); 4) NOD-like receptor signaling pathway ($P = 0.001$); 5) systemic lupus erythematosus ($P = 0.01$); and 6) chemokine signaling pathway ($P = 0.01$). The inclusion of systemic lupus was attributed to differential expression of the complement C1q complex.

Given the significant enrichment of biological processes, molecular functions, and pathways centrally involved in the

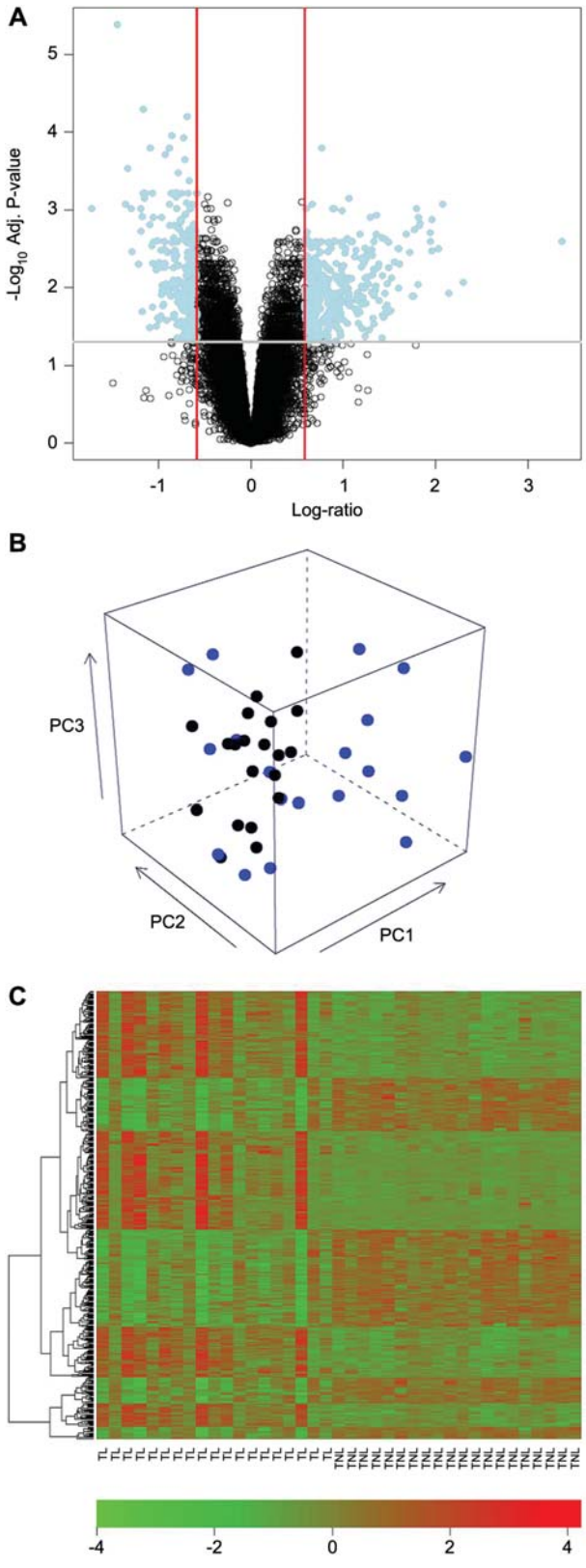

Figure 1 Microarray analysis of the gene expression profiles of myometrium at term not in labor (TNL) and spontaneous term labor (TL).

(A) A Volcano plot showing the ratio between the average gene expression of the term not in labor and term labor groups (X-axis) vs. the significant P-values from the moderated *t*-test. Circles in the upper right and left quadrants represent genes with a fold change >1.5 and a false discovery fate corrected $P<0.05$. With these criteria, 471 genes were differentially expressed between the myometrial transcriptomes of the two groups. (B) Three-dimensional principal component analysis plot (PCA) demonstrating segregation of the TNL and TL groups based on gene expression levels. Black points indicate individual samples from the TNL groups while blue dots represent those from the group with TL. (C) Heatmap of gene expression in TNL and TL clustered by genes. Rows correspond to genes while columns correspond to samples. High expression levels are shown in red, while low expression levels are in green. Data was log (base 2) transformed, and values were mean centered by rows. Color key equals $\log_2$ expression levels.

inflammatory response, we verified differential regulation of genes which are key components of the immune response as well as other genes of interest.

qRT-PCR results

Thirty-one genes were tested for verification of microarray results. Fourteen genes were previously reported in the literature as being related to labor (Table 5A), while 17 genes were selected from among the microarray results (Table 5B).

qRT-PCR confirmed differential expression of 11 genes between the TL and term not in labor (TNL) groups. In addition to genes previously described as upregulated in spontaneous TL [IL-8, IL-6, prostaglandin-endoperoxide synthase 2 (PTGS2/COX2), and CCL2], differential expression of a set of genes not previously described was confirmed. Novel genes with confirmed over-expression during spontaneous TL included: [heparin-binding epidermal growth factor (EGF)-like growth factor (HBEGF)], leukocyte immunoglobulin-like receptor, subfamily A, member 5 (LILRA5), chemokine (C-X-C motif) ligand 6 (CXCL6), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta (NFKBIZ), and SOCS3. Novel downregulated genes included FK506 binding-protein 5 (FKBP5) and aldehyde dehydrogenase 2 (ALDH2) (see Figure 3). Of note, eight of the 11 genes participate in the innate immune response.

Overall, the direction of change in gene expression by PCR was consistent with the microarray analysis in 29 out of 30 genes (96.7%). The comparison of microarray and PCR data for each gene is described in Table 6.

ELISA and immunoblotting

Consistent with the microarray and qRT-PCR data, the median protein concentrations of CCL2 and IL-6 were higher in TL compared to TNL myometrium (Figure 4). While the median concentration of IL-8 was higher in labor, this difference did not reach statistical significance [2.14 pg/mL interquartile range (IQR) 0–52.7 vs. 21.97 pg/mL IQR 1.1–389.4; $P=0.33$]. Similar to the microarray and PCR results, the protein abundance of both CXCL6 and SOCS3 was higher in myometrium from women in labor compared to women not in labor as demonstrated by immunoblot (Figure 5).

Table 4A Gene ontology analysis: biological processes enriched in the differentially expressed genes between spontaneous term labor and term not in labor.

Biological process category	Differentially expressed genes/number of total genes in GO term	Adjusted P-value
Inflammatory response	36/257	1.42E-09
Response to wounding	44/371	1.42E-09
Response to external stimulus	55/576	8.95E-09
Defense response	47/458	2.10E-08
Behavior	32/266	5.40E-07
Chemotaxis	21/123	5.66E-07
Taxis	21/123	5.66E-07
Locomotion	35/334	2.24E-06
Locomotory behavior	24/175	2.87E-06
Immune system process	56/723	4.52E-06
Response to stimulus	116/2042	7.27E-06
Response to stress	76/1146	7.55E-06
Regulation of cell proliferation	42/488	1.39E-05
Immune response	42/500	2.54E-05
Multicellular organismal process	134/2592	9.32E-05
Response to chemical stimulus	46/604	9.53E-05

Partial list (total of 103 significant processes).

Table 4B Gene ontology analysis: molecular functions enriched in the differentially expressed genes between spontaneous term labor and term not in labor.

Molecular function category	Differentially expressed genes/number of total genes GO term	Adjusted P-value
Cytokine activity	22/135	6.02E-07
Heparin binding	11/63	0.0025
Receptor binding	42/619	0.0029
Chemokine activity	8/35	0.0029
Chemokine receptor binding	8/36	0.0029
Glycosaminoglycan binding	12/85	0.0030
G-protein-coupled receptor binding	10/61	0.0030
Polysaccharide binding	12/87	0.0030
Carbohydrate binding	20/228	0.0075
Pattern binding	12/101	0.0104
Growth factor activity	13/123	0.0167
Cytokine binding	10/81	0.0220
Oxidoreductase activity	5/21	0.0287
Cadmium ion binding	3/6	0.0332
NADPH:quinone reductase activity	2/2	0.0434
Thyroxine 5′-deiodinase activity	2/2	0.0434
Interleukin-8 receptor activity	2/2	0.0434
Oxidoreductase activity NAD or NADP as acceptor	4/15	0.0479

Discussion

Principal findings of this study

1) The myometrial transcriptome of women in spontaneous labor at term was dramatically different from that of women not in labor; 471 genes were differentially expressed between the groups; 2) gene ontology analysis indicated specific biological processes (e.g., inflammatory response, chemotaxis, and immune response) and molecular functions (e.g., cytokine activity, chemokine activity, chemokine receptor binding) associated with spontaneous TL; 3) pathway analysis identified six pathways, all involved in inflammation, enriched in the myometrial transcriptome of labor; 4) a novel set of inflammation-related genes differentially expressed in human myometrium during labor was identified including LILRA5, CXCL6, NFKBIZ, and SOCS3 as well as additional genes not previously reported to be involved in term human parturition (FKBP5, HBEGF, and ALDH2); and 5) overall, the process of spontaneous term parturition is characterized by a molecular signature consistent with overexpression of genes involved in inflammation and leukocyte chemotaxis.

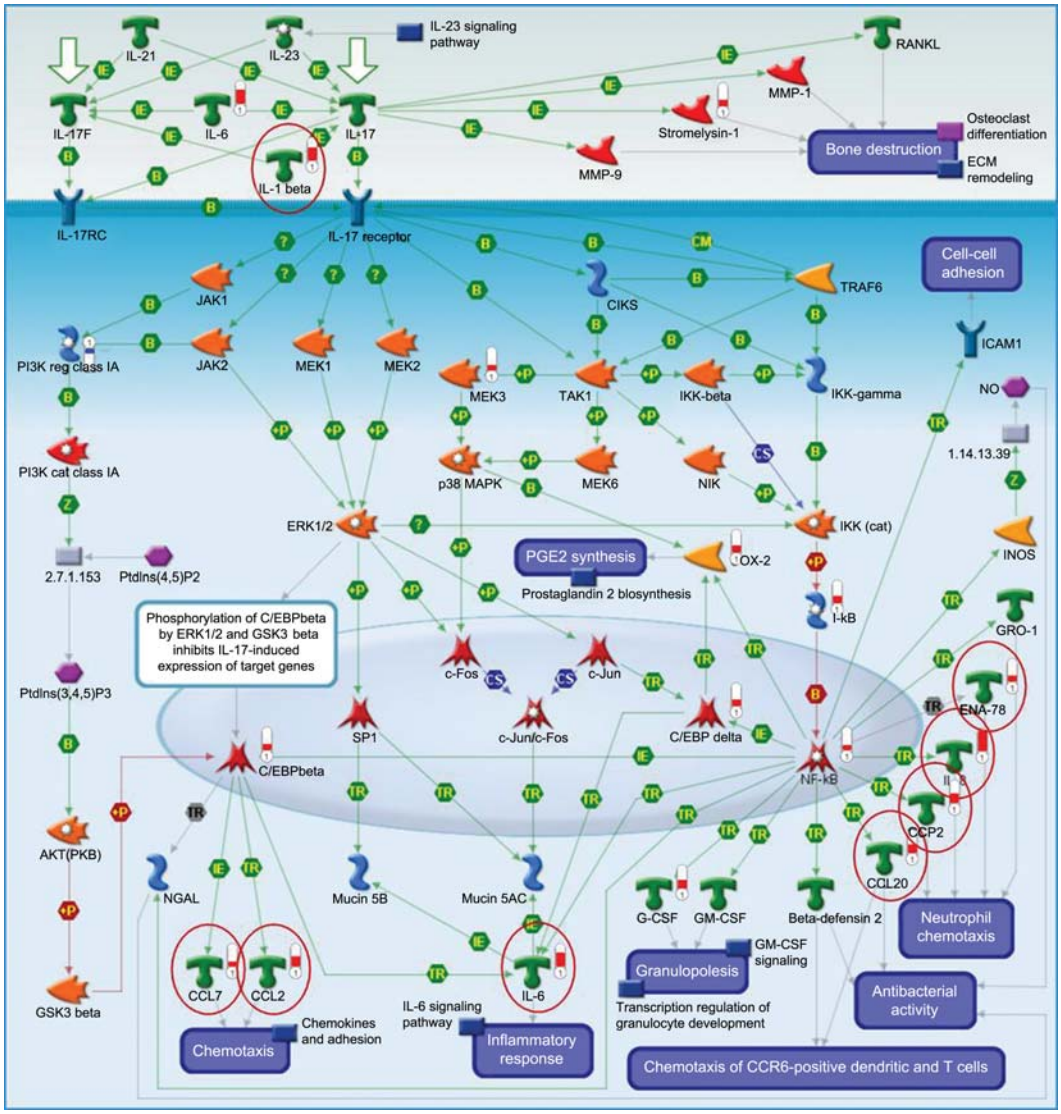

Figure 2 The interleukin-17 signaling pathway (MetaCore).
Display of differentially expressed genes in human myometrium during term labor mapped on the MetaCore interleukin-17 signaling pathway. Red thermometers indicate gene upregulation in labor while blue thermometers indicate downregulation in labor. Of note, the majority of differentially regulated genes in this pathway are those involved in inflammation and chemotaxis (circled genes).

Inflammation: an integral component of labor at term in the myometrium

Inflammation has been implicated in key biological processes required for reproduction, including ovulation, implantation [63, 97, 203] and parturition [14, 15, 17, 31, 130, 137, 160, 173, 189, 206, 240–243, 249, 251, 255–258, 261, 277, 326]. Implantation of the blastocyst generates a pro-inflammatory response within the decidua (TH1-like polarization) [63, 97, 203]. After establishment of placentation, inflammation retreats, allowing a telerogeneic state required for the growth and development of the conceptus. This has been referred to as a TH2-like polarization present within the uterus and the maternal systemic circulation during most of pregnancy. Spontaneous parturition represents the re-emergence of this inflammatory state (TH1 polarization) [121, 251]. Indeed, leukocyte infiltration of human myometrium has been described as a hallmark of the development of the uterus from a quiescent to a contractile organ [309]. Interestingly, gradients of leukocyte infiltration into the uterus and the uterine cervix [218] during labor have been reported. Thomson et al. [309] first described the region-specific leukocyte subpopulations in the fundal and lower uterine segments. The authors reported an increased density of neutrophils and macrophages in the myometrium of both regions during labor, while an increased density of T-cells was limited to the lower uterine segment. Overall, the inflammatory infiltrate was predominant in the lower uterine segment. However, the mechanisms responsible for leukocyte infiltration (chemokine signaling) and the biological processes induced by leukocytes in the uterus are still poorly elucidated.

Table 5A Description of genes selected for qRT-PCR based upon previous reports and results of each gene in current microarray study.

Symbol	Gene name	Function	Microarray results in labor
OXTR	Human oxytocin receptor	The receptor for the hormone and neurotransmitter oxytocin	N/A
THBS1	Thrombospondin 1	Secreted protein associated with the extracellular matrix	Over-expression
SOD2	Superoxide dismutase 2	Mitochondrial enzyme, antioxidant	Over-expression
GJA1	Gap junction protein, alpha-1	Connexin 43, intercellular communication	NS
IL-8	Interleukin-8	Mediates chemotaxis and activation of neutrophils	Over-expression
PTGS2	Prostaglandin-endoperoxide synthase 2	COX 2, regulation of prostaglandin synthesis	Over-expression
CCL2	Chemokine (C-C motif) ligand 2	Monocyte chemoattractant	Over-expression
IL-6	Interleukin-6	Cytokine mediator of acute phase response	Over-expression
IL-1B	Interleukin-1-β	Stimulation of T cells, enhanced proliferation of B cells, proinflammatory	Over-expression
NAMPT	Nicotinamide phosphoribosyltransferase	Visfatin: adipocytokine with anti-apoptotic functions	Over-expression
PTGES	Prostaglandin E synthase	Enzyme catalyzing production of prostaglandin E from prostaglandin H2	Over-expression
ESR1	Estrogen receptor-alpha	Nuclear receptor	NS
HSP90B1	Heat shock protein 90 kDa beta	Molecular chaperone protein required for the proper functioning of steroid receptors	NS
S100A8	S100 calcium binding protein A8	Innate immune response, expressed by macrophages	Over-expression

qRT-PCR = quantitative reverse transcriptase-polymerase chain reaction, NA = not available, NS = not significant.

Table 5B Description of genes selected for qRT-PCR based upon microarray results.

Symbol	Gene name	Function
HBEGF	Heparin-binding EGF-like growth factor	Mitogen for fibroblasts and smooth muscle growth; anti-apoptotic
LILRA5	Leukocyte immunoglobulin-like receptor, subfamily A, member 5	Leukocyte IG-like receptor on monocyte surface, induces secretion of pro-inflammatory cytokines
CXCL6	Chemokine (C-X-C motif) ligand 6	Neutrophil chemoattractant
NFKBIZ	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	Regulation of NF-κB, induction of IL-6 secretion
FKBP5	FK506 binding protein 5	Progesterone receptor-associated immunophilin required for functionally mature steroid receptor
SOCS3	Suppressor of cytokine signaling 3	Inhibition of IL-6, IL-10, and interferon-gamma
ALDH2	Aldehyde dehydrogenase 2 family	Mitochondrial enzyme required for acetaldehyde metabolism
IER3	Immediate early response 3	Cellular resistance to apoptosis
ALDH7A1	Aldehyde dehydrogenase 7 family, member A1	Detoxification of aldehydes generated by lipid peroxidation
HIF1A	Hypoxia inducible factor 1, alpha subunit	Transcription factor with roles in systemic and cellular responses to hypoxia
HOXA11	Homeobox A11	Extracellular matrix metabolism inducing collagen III expression
IL-24	Interleukin-24	Pro-apoptotic cytokine, member of IL-10 superfamily
PSAT1	Phosphoserine aminotransferase 1	Progesterone-induced protein
MMP10	Matrix metallopeptidase 10	Degrades proteoglycans and fibronectin
PROK2	Prokineticin 2	Output molecule from the suprachiasmatic nucleus circadian clock
ALDH1A3	Aldehyde dehydrogenase 1 family, member A3	Retinoic acid synthesizing enzyme
EXOG	Endo/exonuclease (5′-3′), endonuclease G-like	Mitochondrial enzyme involved in programmed cell death

qRT-PCR = quantitative reverse transcriptase-polymerase chain reaction.

Previous investigators have described the increased expression of chemotactic factors which may, in part, account for this accumulation of leukocytes in the uterus [259, 264]. IL-8 is a major chemokine that mediates neutrophil chemotaxis and activation. Increased expression of IL-8 has been reported in gestational tissues [218, 241, 305] and the cervix [132, 219, 272, 275, 276] in spontaneous labor. Our finding that IL-8 mRNA expression is dramatically over-expressed in human myometrium with spontaneous labor is consistent with these reports. These observations are consistent with our 1991 report demonstrating that the median amniotic fluid concentration of IL-8 was higher in women with spontaneous labor at term without infection/inflammation than in those not in labor at term [241]. It is noteworthy that IL-8 production by intrauterine tissues is regulated by progesterone [154]. Thus, a suspension of progesterone action as term approaches may be responsible for the increased production of IL-8, neutrophil infiltration and activation, and the subsequent inflammatory phenotype of the myometrium.

IL-6 mediates the acute phase response and functions as a myokine produced by contracting muscle [96]. Our results confirmed that there is a significant over-expression of IL-6 in myometrium during labor – this observation is consistent

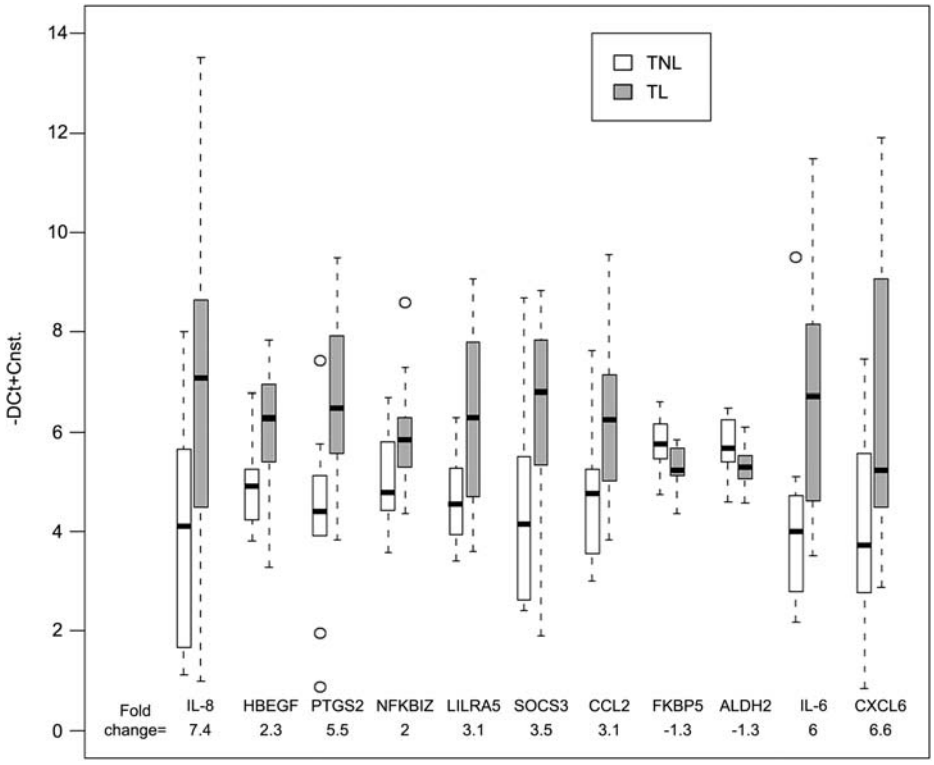

Figure 3 Box plots of significant qRT-PCR assays.
The data is presented as $-\Delta$Ct values (Ct reference gene-Ct target gene) which is a surrogate for gene expression (on a $\log_2$ scale). The boxes encompass 50% of the data from the 1st quartile to the 3rd quartile. The middle line represents the median value (50%) quantile. The whiskers extend to the most extreme data point, but do not exceed values >1.5 times the interquartile range from the box. The circles represent outliers. Significance was defined as a $P<0.05$. TNL = term not in labor, TL = spontaneous term labor, IL = interleukin, HBEGF = heparin binding EGF-link growth factor, PTGS2 = prostaglandin-endoperoxide synthase-2, NFKBIZ = nuclear factor of kappa light chain gene enhancer in B-cells inhibitor zeta, LILRA5 = leukocyte immunoglobulin-like receptor, subfamily A, member 5, SOCS3 = suppressor of cytokine signaling 3, CCL2 = chemokine C-C motif ligand 2, FKBP5 = FK506 binding-protein 5, ALDH2 = aldehyde dehydrogenase, CXCL6 = chemokine C-X-C motif ligand 6.

with the findings of Osman et al. [218]. Furthermore, the current report provides evidence that IL-6 protein is elevated in the myometrium of women in labor at term, compared with women at TNL. These findings are also consistent with our report that IL-6 increases in the amniotic fluid of women with spontaneous labor at term when compared to that of women at TNL [273]. Such observation (made in 1991) has subsequently been confirmed as the expression of IL-6 increases in gestational tissues [128, 132, 218, 306, 337]. This cytokine has become a valuable marker in the assessment of patients with preterm labor and preterm PROM, as well as in the definition of the fetal inflammatory response syndrome (which is associated with the onset of preterm labor) [118, 121, 239, 250, 251, 260, 265, 266].

Some pro-inflammatory cytokines also promote uterine contractility by inducing the expression of PTGS2/COX2, the rate-limiting step of prostaglandin biosynthesis [5, 10, 67, 71, 94, 98, 126, 131, 153, 231, 232]. For example, we and others have demonstrated that IL-1β, TNFα and IL-6 can increase prostaglandin production by amnion, decidua and myometrium [11, 19, 20, 70, 73, 138–140, 155, 175, 198, 199, 202, 294, 325]. PTGS2/COX2 is a rapidly inducible enzyme with reported increased expression in association with labor in myometrium [5, 6, 44, 54, 67, 115, 136, 233, 278, 284, 289, 306] and the uterine cervix [26, 67, 132, 186, 230, 297, 313, 330, 331]. Our results are consistent with these findings.

We have previously reported that the concentration of CCL2 (also known as MCP-1) is increased in the amniotic fluid of women in spontaneous labor at term compared to those not in labor, including spontaneous preterm delivery [92, 93]. Herein, we describe significant over-expression of CCL2, a monocyte chemoattractant also involved in macrophage activation, in spontaneous term parturition. Esplin et al. [91] described increased expression of CCL2 in human myometrium and the chorioamniotic membranes during labor using microarray analysis and Northern blot analysis. Interestingly, a recent report demonstrated that progesterone attenuates the myometrial expression of CCL2 in pregnant rats, implicating CCL2 as a potential therapeutic target for the prevention of preterm labor [283]. In our analysis, we confirm increased CCL2 mRNA expression in the myometrium during labor and describe increased CCL2 protein concentrations in myometrium from women in spontaneous labor at term, providing further support for the role of this chemokine in the mechanisms of labor.

Table 6 Comparison of qRT-PCR and microarray analysis of select genes.

Gene symbol	P-value qRT-PCR	Fold change qRT-PCR	Direction of change in labor qRT-PCR	Corrected P-value microarray	Fold change microarray	Direction of change in labor microarray
PTGS2*	0.004	5.47	↑	0.003	3.86	↑
HBEGF*	0.011	2.32	↑	0.010	2.56	↑
CCL2*	0.015	3.08	↑	0.013	3.40	↑
LILRA5*	0.016	3.09	↑	0.001	3.48	↑
IL-8*	0.022	7.38	↑	0.003	10.36	↑
IL-6*	0.011	6.01	↑	0.009	4.92	↑
CXCL6*	0.028	6.60	↑	0.005	2.95	↑
NFKBIZ*	0.027	2.04	↑	0.007	2.69	↑
FKBP5*	0.038	−1.34	↓	0.000	−1.59	↓
SOCS3*	0.040	3.46	↑	0.006	2.52	↑
ALDH2*	0.046	−1.31	↓	0.007	−1.52	↓
IER3	0.066	1.91	↑	0.006	2.55	↑
SOD2	0.075	1.79	↑	0.012	2.74	↑
ALDH7A1	0.086	−1.27	↓	0.008	−1.54	↓
IL-1B	0.093	3.15	↑	0.012	4.43	↑
NAMPT	0.096	2.00	↑	0.002	2.68	↑
HSP90B1	0.135	1.25	↑	0.143	1.17	↑
HIF1A	0.170	1.34	↑	0.014	1.62	↑
S100A8	0.187	1.88	↑	0.002	3.85	↑
HOXA11	0.206	−1.23	↓	<0.001	−1.81	↓
IL-24	0.206	2.73	↑	0.018	2.75	↑
PSAT1	0.219	1.92	↑	0.010	1.67	↑
PTGES	0.237	1.77	↑	0.013	3.36	↑
MMP10	0.331	1.71	↑	0.020	2.87	↑
GJA1	0.394	1.09	↑	0.405	1.17	↑
PROK2	0.406	1.19	↑	0.003	3.89	↑
ESR1	0.419	1.04	↑	0.964	1.01	↑
ALDH1A3	0.497	1.00	↑	0.004	2.29	↑
EXOG	0.830	−1.20	↓	0.001	3.90	↑
OXTR	0.856	−1.15	↓	NA	NA	NA
THBS1	0.266	1.42	↑	0.016	2.38	↑

Direction of change denotes change in spontaneous term labor.
*Genes with significant results by microarray analysis and confirmed differential expression by qRT–PCR.
NA = Not applicable. A probe set for this gene is not included on the Illumina® HumanHT-12 v3 expression microarray platform.
↑ = increased expression in the term labor group compared to the term not in labor group.
↓ = decreased expression in the term labor group compared to the term not in labor group.

The use of systems biology to delineate the biological processes and pathways characterizing spontaneous term parturition

We report herein that six pathways are enriched in the myometrium during labor. Each of these pathways is central in the deployment of an inflammatory response: cytokine-cytokine receptor interaction, Jak-STAT signaling, the complement and coagulation pathway, NOD-like receptor signaling, systemic lupus erythematosus (specifically the complement C1q complex), and chemokine signaling. Interestingly, components of these pathways have been previously linked to both normal parturition [57, 75, 76, 159, 180, 201, 213, 221, 226–228, 235, 271, 274, 308] and the ''Great Obstetrical Syndromes'' [32, 33, 64, 79–82, 84, 85, 89, 238, 267, 291, 295, 307, 320], including preterm labor [28, 58, 74, 77, 83, 86–88, 119, 120, 164, 165, 211, 223, 245, 252, 254, 290, 292, 293, 316–319]. Indeed, our group has reported an association between activation of the NALP3 inflammasome and the common pathway of parturition [119]. Moreover, we have previously demonstrated that normal pregnancy is characterized by activation of the complement system [235]. The most significantly enriched biological process and molecular function in spontaneous term parturition were the ''inflammatory response'' and ''cytokine activity'', respectively. Novel genes involved in these pathways with over-expression in myometrium during labor all have reported molecular functions in the initiation, maintenance, and regulation of physiologic inflammation including CXCL6, LILRA5, NFKBIZ, and SOCS3.

Chemokines play an integral role in both the innate and adaptive host response as well as immune homeostasis [299, 300]. Induced by IL-1β [332], CXCL6 shares high functional homology with IL-8. A potent neutrophil chemoattractant, over-expression of CXCL6 has been reported in the chorioamniotic membranes during TL [128]. However, unlike the closely related IL-8, amniotic fluid concentrations of CXCL6 do not significantly change with the onset of TL,

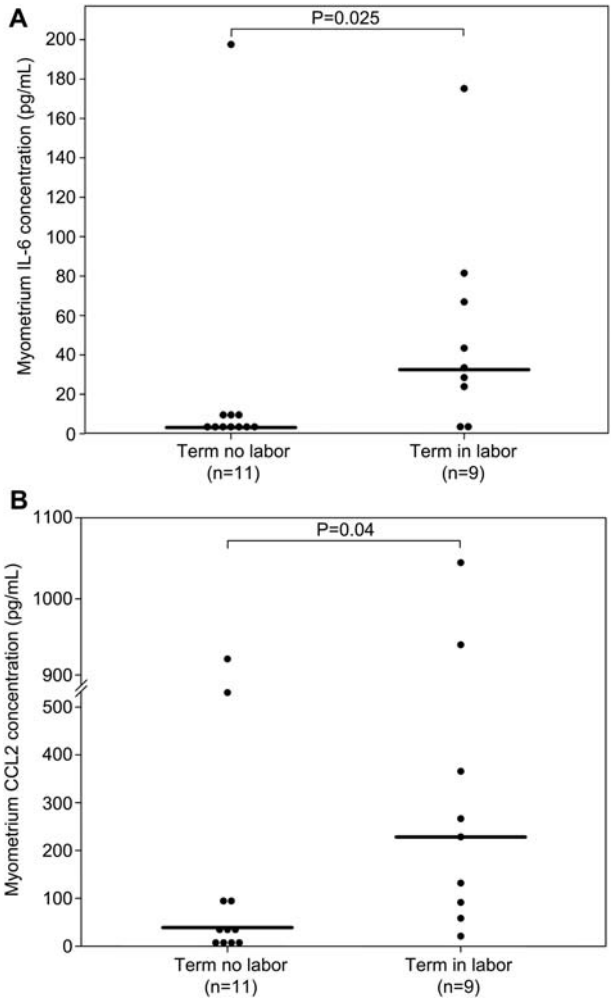

Figure 4 Comparison of myometrial protein concentrations of interleukin-6 and CCL2 between term not in labor and spontaneous labor.
(A) The median interleukin-6 concentration was significantly higher in women at term in labor compared to those without labor [term not in labor 1.87 pg/mL interquartile range (IQR), IQR 0–9.0 vs. term labor 34.59 pg/mL, IQR 14–73; P = 0.25]. (B) Median CCL2 protein concentration was also higher in myometrium from women in labor compared to those not in labor (term not in labor 35.8 pg/mL, IQR 6–107 vs. term labor 229.41 pg/mL, IQR 74–652; P = 0.04). CCL2 = chemokine C-C motif ligand 2, IL = interleukin.

although CXCL6 concentrations in amniotic fluid are higher in women with preterm labor compared to those with preterm gestation who eventually deliver at term [200]. Herein, we report the novel findings that CXCL6 is expressed by human myometrium and CXCL6 mRNA and protein expression are significantly increased in human myometrium during spontaneous TL in the absence of histologic chorioamnionitis. Indeed, the only gene with a more profound increase in gene expression was IL-8. Interestingly, CXCL6 has been described to interact synergistically with CCL2 to increase neutrophil infiltration 10-fold in gastrointestinal tumors [116]. The enhanced expression of CXCL6 might strongly contribute to the myometrial infiltration by leukocytes observed during labor.

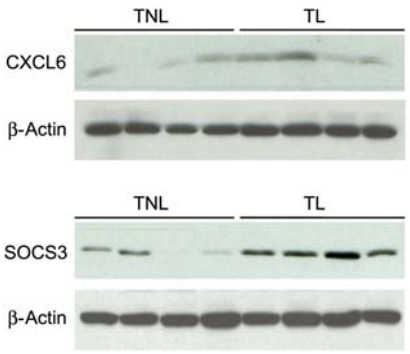

Figure 5 Immunoblotting analysis of CXCL6 and SOCS3 in term myometrium.
Ten μg of total proteins were electrophoresed in 18% and 12% SDS-PAGE for CXCL6 and SOCS3, respectively. Protein concentrations of both CXCL6 and SOCS3 were higher in TL specimens compared to TNL. TNL = term not in labor, TL = term labor, CXCL6 = chemokine C-X-C motif ligand 6, SOCS3 = suppressor of cytokine signaling 3.

Leukocyte immunoglobulin-like receptor subfamily A, member 5 (LILRA5; LIR9) is a member of the family of leukocyte immunoglobulin-like receptors. To date, the expression of LILRA5 in reproductive tissues has not been reported. First described in 2003 [18], this gene has been implicated in the early activation of the innate immune response. Cross-linking of LILRA5 molecules on the surface of monocytes induces a calcium flux resulting in secretion of the pro-inflammatory cytokines IL-1β, IL-6 and TNFα [18]. Given that IL-1β has been implicated in the initiation of myometrial contractions [7, 60, 218], the induction of IL-1β secretion by LILRA5 suggests a role for this novel receptor in the onset of labor. While the precise role of LILRA5 in the process of human parturition remains to be elucidated, its increased expression in human myometrium during spontaneous labor is a new lead for the study of human parturition.

Another pivotal regulator of IL-6, NFKBIZ, was overexpressed in human myometrium during labor. NFKBIZ is a nuclear IκB protein which maintains dual roles in the regulation of NF-κB with both positive and negative effects [187, 204]. The mechanisms determining the opposite actions are currently unknown, but are hypothesized to be cell-type specific [161, 335]. Interestingly, recent studies in a knock-out mouse model have demonstrated that NFKBIZ expression is required for IL-6 production. Macrophages from NFKBIZ knock-out mice had a profoundly impaired ability to produce IL-6 in response to LPS, IL-1, and TLR ligands [334]. Moreover, suppression of NFKBIZ by small interfering RNA significantly decreased monocyte IL-6 production in response to LPS [279]. Given the role of NF-κB in the regulation of immune responses, the involvement of this positive and negative regulator of inflammatory gene expression in human labor warrants further investigation.

SOCS3 is a member of the family of cytokine signaling inhibitors which regulate cytokine signaling through the JAK/STAT pathway. In particular, SOCS3 has been described as the ''central negative regulator'' of macrophage

IL-6 signaling [336]. In mice with a conditional SOCS3 gene deletion in macrophages, IL-6 hyper-responsiveness has been demonstrated [56]. We report SOCS3 over-expression in myometrium during labor. Contradictory data exist regarding the differential expression of SOCS3 in the chorioamniotic membranes as expression after TL has been reported to be both under- [16] and over-expressed [128]. While inflammation characterizes term human parturition, the process would require close regulation to remain physiologic. Therefore, SOCS3 may play an integral role during normal labor to abrogate the potentially damaging effects of an uncontrolled inflammatory response.

Evidence for functional progesterone withdrawal in human parturition

Suspension of progesterone action is believed to be crucial for the initiation of parturition. In most animals, this is mediated by a decrease in circulating maternal progesterone concentrations [99, 184, 185]. Parturition in humans and other primates, however, occurs without a systemic progesterone withdrawal in maternal serum [38, 287, 323]. Nonetheless, a role for progesterone withdrawal as a trigger for parturition is supported by the fact that administration of nuclear progesterone receptor (nPR) antagonists (e.g., RU486) augments myometrial contractility and excitability and initiates labor at all stages of pregnancy [8, 46, 47, 72, 104, 109, 111, 129, 301]. Interruption of progesterone/nPR signaling is sufficient to initiate the full cascade of parturition and therefore, it is generally considered that human parturition involves a functional rather than a systemic progesterone withdrawal whereby myometrial cells become desensitized to relaxatory nPR-mediated actions of progesterone [3, 12, 22, 38, 42, 45, 48, 192–194, 215, 237, 269, 285, 287, 329]. To date, the precise mechanisms for a functional progesterone withdrawal in TL remain to be elucidated. A role for changes in the nPR signaling pathway is supported by the following observations: 1) term parturition is associated with increased expression of inhibitory nPR isoforms in human myometrium [51, 191, 195]; 2) there is decreased expression of nPR co-activators in myometrium at term [52, 152]; and 3) expression of nPR co-repressors increases during human labor [65]. Our microarray data indicate that nPR and nPR co-regulator expression is not different between the TL and not in labor groups. However, this may be due to sensitivity issues as nPR and steroid co-regulator gene expression is relatively low in human myometrium and small changes in gene expression may not be detectable by microarray analysis. Nonetheless, several genes identified as differentially expressed with labor onset provide evidence for decreased nPR transcriptional activity, and therefore functional progesterone withdrawal, in laboring tissue: 1) IL-8, which is decreased by progesterone [154], and whose expression is increased in myometrium during labor; 2) PTGS2/COX2, which is also decreased by progesterone [78], whose expression in myometrium was also increased during spontaneous TL; and 3) the immunophilin (FKBP5; alias FKBP51), whose expression is normally increased by progesterone [144], but was decreased in the TL group. Recently, the differential expression of FKBP5 has been described in cultured human chorion and decidua cells (obtained from women at TNL) treated with progesterone [207]. While FKPB5 expression increased in the chorion with exogenous progesterone treatment, its expression decreased in the decidua.

This is the first study reporting decreased expression of the immunophilin FKBP5 [286] in myometrium from women at term in labor compared to that obtained from women not in labor. FKBP5 is an immunophilin that participates with other proteins, such as Hsp90 and p23 in forming the mature steroid hormone receptor complex capable of binding steroid with high efficacy and affinity [209, 229, 288]. Of note, FKBP52 (alias FKBP4; related immunophilin) null mice exhibit decreased uterine responsiveness to circulating progesterone leading to implantation failure [314]. However, the pregnancies of null mice with a specific genetic makeup (CD1 null mice) could be rescued with progesterone supplementation. Interestingly, the concentrations of progesterone treatment required to maintain pregnancy were specific to the stage of pregnancy-increasing concentrations were required as the pregnancy progressed [314]. Importantly, FKBP5 has been described to preferentially accumulate in the mature nPR complex [9, 208], has a competitive advantage for progesterone receptor association over both the FKBP52 and Cyp-40 immunophilins [9], and its expression is upregulated by progesterone via the type-B nPR (PR-B) in breast cancer cell models [144, 145]. In this context, decreased expression of this PR-B-responsive gene may reflect decreased PR-B-mediated progesterone activity in the myometrial cells. This is important because PR-B is thought to be the principal mediator of relaxatory actions of progesterone in human pregnant myometrium [51, 52, 191, 195]. Therefore, a decrease in its transcriptional activity may reflect functional progesterone withdrawal. An alternative hypothesis is that decreased FKBP5 limits the capacity for progesterone to maintain a relaxed phenotype via PR-B since it participates as a molecular chaperone in the formation of functional PR-B complexes with high affinity for ligand. Therefore, the decreased myometrial expression of FKBP5 in association with TL may not only reflect functional progesterone withdrawal but may also be a novel mechanism for functional progesterone withdrawal by limiting progesterone actions via PR-B. Further studies are needed to test these hypotheses.

Additional genes associated with spontaneous labor at term

We report the novel finding of HBEGF over-expression in myometrium during labor compared to quiescent myometrium. This is consistent with evidence that exercise is associated with increased expression of HBEGF in skeletal muscle [105]. Of interest, in a rat model, the closely related epidermal growth factor (EGF) produced rhythmic uterine contractions that were abolished when tissue was treated with anti-EGF antibodies [107]. It is therefore plausible that HBEGF may also contribute to uterine contractility. HBEGF also acts as a mitogen for smooth muscle and fibroblasts while protecting against apoptosis during stress [141]. A transgenic mouse model demonstrated that increased expres-

sion of HBEGF is associated with increased glucose uptake and insulin sensitivity consistent with facilitation of glucose disposal [105]. During pregnancy, HBEGF expression has been associated with trophoblast survival [4, 150, 167, 328] and we have previously reported decreased HBEGF placental expression in preeclampsia [168].

ALDH2, which was under-expressed during labor, is a mitochondrial enzyme essential for the oxidation and subsequent elimination of acetaldehyde [162]. The pathway of acetaldehyde metabolism interacts with that of retinol metabolism; interestingly, retinoic acid decreases transcription of the progesterone receptor gene [49] and increases expression of the oxytocin receptor gene in rat myometrium [166]. Indeed, gene deletion for retinoic acid receptor-α is associated with a decreased ethanol and acetaldehyde clearance with decreased activity of ALDH2 [127]. Myometrial ALDH2 expression has not been previously reported. The precise role of this gene in human parturition warrants further investigation.

Comparison with previous reports of functional genomics in the study of term human parturition

Aguan et al. [1] first reported the use of functional genomics to address the molecular mechanisms of labor in humans employing cDNA macroarrays to assess the expression of 588 genes from the myometrium of women with (n = 3), and without TL (n = 3). Differential expression was observed in 21 genes of diverse functions. These results were not confirmed with a targeted approach (Northern blot or PCR). Using suppression subtractive hybridization (SSH), Chan et al. [39] compared myometrium from women at TNL and those with dysfunctional labor. The authors identified 400 clones. Thirty clones were differentially expressed and over-expression of cyclophilin, SOD2, and IL-8 was confirmed in patients with dysfunctional labor using qRT-PCR. We found over-expression of SOD2 and IL-8 in our microarray data. However, confirmatory qRT-PCR was significant only for IL-8. The molecular basis of dysfunctional TL including arrest of dilatation and arrest of descent is an area for future study.

Havelock et al. [136] employed cDNA microarray in their analysis comparing spatial differential gene expression in myometrium from women with and without labor with pooled samples of four specimens per group. Differential expression of PTGS2/COX2 and calgranulin B (S100A9) was confirmed with qRT-PCR. In the current study, both PTGS2/COX2 and S100A9 were upregulated according to the microarray analysis, with confirmed over-expression of PTGS2/COX2. Using cDNA microarray, Esplin et al. [90] reported the differential expression of 56 unique genes involved in human parturition (TNL, n = 5, TL, n = 5). The authors confirmed increased expression of four genes in labor: THBS1, SOD2, PBEF1, and NNMT. Our microarray findings were consistent with these results. In contrast, verification of these findings using qRT-PCR in an independent set of samples did not yield significant results for THBS1, SOD2, or PBEF1. The authors also noted over-expression of CCL2 in myometrium during labor and confirmed this finding in a subsequent investigation [91].

Bukowski et al. [25] reported results describing the differences in transcriptomes among the uterine fundus, lower uterine segment, and cervix as they vary prior to (n = 6) and during labor (n = 7). However, after correction for multiple comparisons, the differences were no longer significant [262]. Validation studies with RT-PCR did confirm decreased expression of repressor of estrogen receptor alpha (REA) and retinoid X receptor alpha (RXR) in the uterine fundus during labor. Our findings in the present study could not confirm differential expression of REA or RXR in myometrium from the lower uterine segment of women in labor. Recently, the group of O'Brien et al. [214] utilized the Applied Biosystems Genome Survey Microarray version 2 to investigate the myometrial transcriptome of labor. A total of 698 genes were differentially expressed between the TL (n = 3) and TNL (n = 3) groups. Over-expression of PSCDBP, EDNRB, TLR2, FLJ35383, TWIST1, and RGS12 was reported. Of interest, none of these genes were significantly overexpressed in the current study.

While differential gene expression in myometrium from women with and without labor has been described using global methods and targeted approaches, such as Northern blot analysis and qRT-PCR, differences in detection between methods are frequently noted, as demonstrated above. Similarly, overlap between different reports is also evident. The lack of uniform gene expression signatures is possibly due to differences in patient selection, experimental design, statistical analysis and the platforms used.

A noteworthy aspect of this study (for future in-depth functional analyses of the differentially expressed genes) is our finding that some differentially expressed genes in Tables 2 and 3 do not have a known function. Conserved among mammals, LOC100132684, synonymous with C14ORF132, (Table 3) encodes a novel 83-aa protein with no known domains. C5ORF4, encoding a 333-aa protein without a known function, is even more highly conserved throughout the animal kingdom. Our understanding of C5ORF4 may benefit from the annotation efforts of diverse organismal genome projects, as the mosquito (*Aedes aegypti*) homolog of this gene appears to be the first member of this family to be functionally annotated as a sterol desaturase. These results indicate that some platform annotations need updating or further interpretation aided by GenBank synonyms and homology searches to derive functional meanings.

Several differentially expressed loci in our study portray evidence of genomic complexity beyond protein-coding genes. In particular, STRBP (Table 3) hosts a small regulatory microRNA, miR-600, in its 3′ untranslated region. Therefore, the functional regulatory output of this locus may not be limited to the STRBP protein, and may include downstream effects of the microRNA. We have previously reported differential expression of microRNAs in the uterine cervix during TL [133]. Additionally, HIF1A and HOXA11 both have endogenous non-coding cis-antisense RNA transcripts [40, 268]. Understanding the impact of functional RNA on the regulation of genes in TL should aid our effort to place these genes into regulatory networks.

Strengths and limitations of the study

A major strength of this study is the large sample size included in the microarray analysis: the largest reported to date. In addition, the results of the microarray experiments for selected genes were confirmed with qRT-PCR in an independent set of samples (biological validation). Moreover, confirmation of differential protein expression between the groups was performed using a third separate group of specimens. We have identified novel genes previously unrecognized to participate in human labor and verified the differential expression of genes previously implicated in the transformation of human myometrium from a quiescent to a contractile organ.

Our results also include the first description of the possible biological processes, molecular functions, and pathways associated with the transformation of human myometrium from a quiescent to a contractile organ derived from an unbiased and comprehensive analysis of the myometrial transcriptome. These findings provide a basis for future studies in which the differences and similarities in the myometrial transcriptome between women in term and preterm labor can be addressed. In those future studies, it may be helpful, for greater understanding of gene regulation in labor as well as for development of therapeutics, to define the high-level network regulators governing the entire functional module of genes emerging as different in TL vs. non-labor myometrium. Integration of genome-wide chromatin immunoprecipitation and sequencing (ChIP-seq) results from existing public studies of transcription factors involved in the processes we identified should help test the hypothesis that such transcription factors are master regulators. As a proof of principle, we asked whether any known human genes that are direct targets of NF-κB by ChIP-ditag sequencing [178] are differentially expressed in TL. In fact, 10 differentially expressed genes from our study (ENC1, PDE4B, IL-8, PIM1, RGS10, SOD2, IL8RB, IL-1B, NFKBIZ, and IER3) are genomic direct targets of the RelA NF-kB subunit [178].

A potential shortcoming of our study is the racial polarity of our patient population, which is mainly African-American. The generalizability of our findings to other patient populations will require future investigation. Also, our results specifically describe the stereotypic transcriptome of myometrium from the lower uterine segment of the uterus and do not address the concurrent changes in the transcriptome of the uterine fundus during spontaneous labor at term. Furthermore, the relationship between differential expression of genes in the myometrium and the subsequent onset of labor cannot be studied using serial sampling in women. These studies would be important to establish causality, and would need animal experimentation.

In conclusion, spontaneous TL is characterized by a stereotypic myometrial transcriptome including 471 differentially expressed genes. The application of high-dimensional biology techniques (transcriptomics) has enabled the identification of differentially expressed genes and processes involved in human parturition, and demonstrated the strong association between spontaneous labor and inflammation. These studies are essential for the understanding of parturition and will serve as the basis for understanding the differences between normal labor at term and dysfunctional labor.

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Supplementary material

Materials and methods

Patient selection and clinical definitions

Patients not in labor underwent a cesarean section secondary to a fetus in non-cephalic presentation, previous uterine surgery or classical cesarean section, or an elective cesarean section with no more than one previous cesarean section. Only women who delivered an appropriate for gestational age neonates (AGA) were included. Women in spontaneous labor underwent cesarean section due to a fetal malpresentation or for non-reassuring fetal status as determined by the clinical staff. Patients with clinical or histological chorioamnionitis, those undergoing induction of labor, and those who underwent cesarean section for arrest of dilatation or arrest of descent were excluded.

Labor was diagnosed in the presence of spontaneous regular uterine contractions occurring at a minimum frequency of 2 every 10 min with cervical change that required hospital admission. Histologic chorioamnionitis was diagnosed using previously described criteria [4, 5]. Clinical chorioamnionitis was diagnosed using the criteria proposed by Gibbs et al. [3] An AGA neonate was defined as a birthweight between the 10^{th} and 90^{th} percentile for the gestational age at birth [1]. Arrest of dilatation was defined as entry into spontaneous labor without further cervical change after 2 h, despite adequate contractions. The diagnosis of an arrest of descent was made in patients with complete cervical dilation without continued fetal descent after >1 h.

Microarray analysis

In brief, after purification of RNA using an RNeasy Mini Kit, 500 ng of total RNA was amplified and biotin-labeled with the Illumina® TotalPrep RNA Amplification Kit (Ambion, Austin, TX, USA). Labeled cRNAs were hybridized to the Illumina HumanHT-12 v3 expression BeadChip and imaged using a BeadArray Reader. Raw data was obtained with BeadStudio Software V.3.4.0 (Illumina).

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

Briefly, 0.2X pool of specific gene expression assays (Applied Biosystems, Foster City, CA, USA) was used as the source of primers. Preamplification reactions contained 1.25 μL cDNA, 2.5 μL TaqMan PreAmp master mix (Applied Biosystems) and 1.25 μL pooled assay mix. The reaction was performed with a thermal cycler for 14 cycles at 95°C for 15 s and 60°C for 4 min. After cycling, the reaction was diluted 1:5 with ddH_2O to a final volume of 25 μL. Fluidigm 96.96 Dynamic Array chip was used to perform the next step qRT-PCR assays. The 96.96 array chip was primed in an Integrated Fluidic Circuit (IFC) controller. After priming, 2.5 μL 20X TaqMan gene expression assays (Applied Biosystems) were mixed with 2.5 μL 2X assay loading reagent (Fluidigm) and loaded into the assay inlet on the 96.96 array chip. 2.25 μL preamplified cDNA was mixed with 2.5 μL TaqMan Universal PCR master mix (Applied Biosystems) and 0.25 μL 20X sample loading reagent (Fluidigm) and loaded into the sample inlet on the chip. The chip was returned to the IFC controller for loading. After loading the samples and assays, the chip was placed into the Biomark System to run the reactions.

Enzyme-linked immunosorbent assay

Snap-frozen myometrium tissue was liquid nitrogen pulverized using a mortar and pestle. Protein lysates were obtained using RIPA buffer (Sigma-Aldrich, St. Louis, MO, USA) containing Complete Mini Protease Inhibitors (Roche Diagnostics, Indianapolis, IN, USA) and kept at –80°C until analysis. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA).

Immunoblotting

Specimens from eight cases in each group were included for immunoblot. Ten μg of total protein was electrophoresed in 18% or 12% SDS-PAGE under reducing conditions. Proteins were then electro-transferred onto PVDF membranes and blocked in 5% milk in TBST for 1 h at room temperature and incubated with a murine monoclonal anti-SOCS3 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or with a goat polyclonal anti-CXCL6 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After four washes in TBST, membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h and chemiluminescence was detected with Chemi Glow (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis

Microarray

The Illumina BeadStudio software suite was used to extract raw gene expression values from the array images. Data quality was assessed based on Illumina's positive and negative control probes on each array and by inspection of the distributions of probe intensities. Data was normalized using the quantile normalization method [2]. Probes that were present (detection $P<0.1$) in at least five samples were retained for further analysis. The moderated *t*-test was implemented in the *limma* [6] library of Bioconductor.

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