Signature pathways identified from gene expression profiles in the human uterine cervix before and after spontaneous term parturition

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OBJECTIVE: This study aimed to discover "signature pathways" that characterize biologic processes, based on genes differentially expressed in the uterine cervix before and after spontaneous labor.

STUDY DESIGN: The cervical transcriptome was characterized previously from biopsy specimens taken before and after term labor. Pathway analysis was used to study the differentially expressed genes, based on 2 gene-to-pathway annotation databases (Kyoto Encyclopedia of Genes and Genomes [Kanehisa Laboratories, Kyoto University, Kyoto, Japan] and Metacore software [GeneGo, Inc, St. Joseph, MI]). Overrepresented and highly impacted pathways and connectivity nodes were identified.

RESULTS: Fifty-two pathways in the Metacore database were enriched significantly in differentially expressed genes. Three of the top 5 pathways were known to be involved in cervical remodeling. Two novel pathways were plasmin signaling and plasminogen activator urokinase signaling. The same analysis with the Kyoto Encyclopedia of Genes and Genomes database identified 4 significant pathways that the impact analysis confirmed. Multiple nodes that provide connectivity within the plasmin and plasminogen activator urokinase signaling pathways were identified.

CONCLUSION: Three strategies for pathway analysis were consistent in their identification of novel, unexpected, and expected pathways, which suggests that this approach is both valid and effective for the elucidation of biologic mechanisms that are involved in cervical dilation and remodeling.

Key words: cervical dilation, cervical remodeling, cervix, gene signature network, labor, microarray, parturition, pathway analysis, plasmin, systems biology

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★ EDITORS' CHOICE ★

Labor, delivery, and the postpartum period are accompanied by dramatic changes in the uterine cervix.[1-8] Adverse pregnancy outcome in term (arrest of dilation) and preterm (cervical insufficiency[9] and preterm labor) gestation may occur as a result of cervical disease. Thus, insights into the processes that are involved in cervical dilation and remodeling are critical to the understanding of abnormal labor. The current knowledge of the biological functional of the cervix has been derived from the study of human cervical biopsy specimens with hypothesis-driven research.[2,3,10-14] However, the mechanisms that underlie these processes are not completely understood.

Most research on the biology of the uterine cervix during pregnancy has been conducted with a reductionist approach. Reductionism is the study of a phenomenon by identifying the individual components of a system.[15] The assumption is that a complex system can be understood by investigating the units of the whole.[15] In the case of a biological process, these parts may be represented, for example, by the study of individual genes, proteins, carbohydrates, and lipids. Although there has been a great deal of progress made to date with a reductionist approach in physics, biology, and

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TABLE 1
The top 20 pathways*

Map name	Genes in reference array (n)	Genes in differentially expressed list (n)	Adjusted *P* value
Chemokines and adhesion	206	20	3.85E-06
ECM remodeling	60	12	3.85E-06
Plasmin signaling	47	10	1.42E-05
PLAU signaling	46	9	6.64E-05
VEGF-family signaling	45	9	6.64E-05
HGF signaling pathway	43	8	.0003
NAD metabolism	54	8	.0011
Role of AP-1 in regulation of cellular metabolism	43	7	.0016
VEGF signaling via VEGFR2-generic cascades	43	7	.0016
Interleukin-6 signaling pathway	32	6	.0026
FGF-family signaling	34	6	.0031
MIF in innate immunity response	57	7	.0061
Estrone metabolism	14	4	.0064
HETE and HPETE biosynthesis and metabolism	42	6	.0064
Role of PBX in fibroblasts signaling pathways	26	5	.0064
Transcription regulation of amino acid metabolism	42	6	.0064
Interferon gamma signaling pathway	63	7	.0071
PH proteins participation in RTKs adaptor complexes formation and intermediation with focal adhesion complex: part 2	63	7	.0071
Antiapoptotic function of TRADD/TRAF2 complex	31	5	.0096
Prostaglandin 1 biosynthesis and metabolism	17	4	.0096

* Number of genes in the array = approximately 19,886.

medicine, reductionism does not take into account component-component links and the dynamics that result from these interactions. In addition, because a biological function can rarely be attributed to 1 or a few molecules, there is a need to apply a method that can identify and describe the networks of functionally active components (such as proteins, transcription factors, signaling pathways, and metabolic networks) to provide a comprehensive understanding of a physiologic process or disease.[16,17] *Network biology*, defined as a quantifiable description of the networks that characterize various biological systems, can be used to obtain such an understanding.[16]

We have used a systems biology approach to the study of the uterine cervix in labor and delivery. As a first step, the gene expression patterns in cervical tissue before and after term parturition with the use of functional genomics were described.[18,19] Next, we have undertaken an examination of the uterine cervix transcriptome after term labor using Gene Ontology analysis. This analysis identified specific biologic processes and molecular functions as being involved. Examples include inflammation, response to biotic stimulus, and apoptosis.[18] However, merely enumerating the biologic processes and molecular function does not provide information about the interactive and dynamic properties of the putative genes and proteins that are involved.

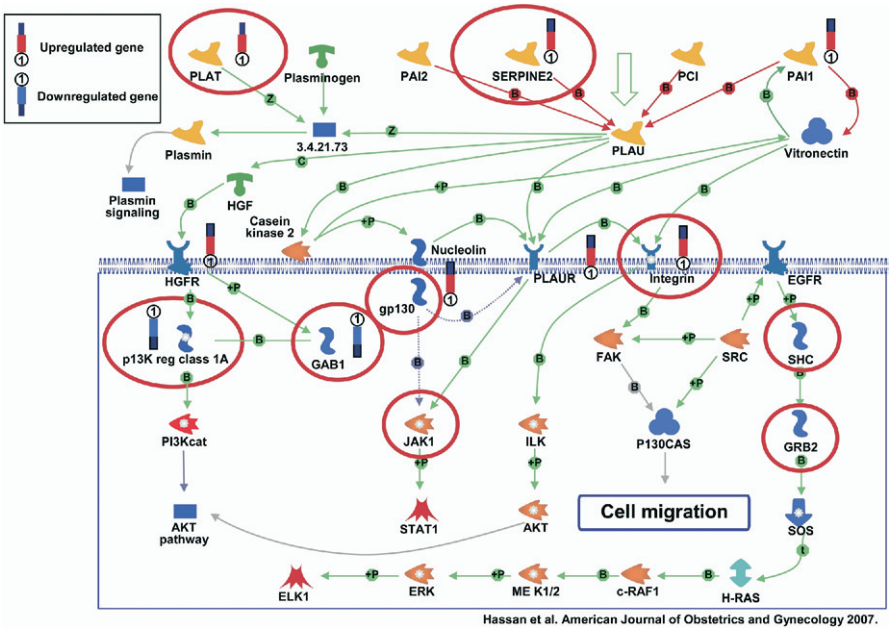
Recent developments in systems biology allow for the construction of maps that display the interaction among genes, proteins, and transcription factors into protein-protein, signaling, metabolic, and transcription-regulatory networks.[20] Thus, the third step in our investigation, the use of network and pathway analysis to identify significant "signature networks," allows for a more comprehensive view of the process of cervical change in labor and delivery. The objective of this study was to identify these signature networks by applying network and pathway analysis to the observed gene expression changes in the uterine cervix in women not in labor at term and in women who underwent spontaneous term labor.

Materials and Methods

Study design

A cross-sectional study was performed in patients who underwent elective cesarean delivery with an unripe cervix (term not in labor) and in patients after spontaneous vaginal delivery (term labor). The cervical transcriptome before and after labor was profiled in cervical biop-

FIGURE 1
Display of differentially expressed genes in the uterine cervix after term spontaneous labor mapped on the PLAU signaling pathway



The *thermometer* represents differentially expressed gene (*red* indicates upregulated; *blue* indicates downregulated). Connectivity analysis results: Several nodes in the PLAU signaling pathway were found to be significant in providing connectivity among the differentially expressed genes. Significant nodes are encircled in *red*.

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sies (before labor [n=7] and after labor [n=9]), using Affymetrix GeneChip microarrays (HG-U133 Plus 2.0; Affymetrix Inc, Santa Clara, CA). The details of this study have been reported previously.[18]

Pathway analysis

To identify pathways relevant to cervical biology during parturition, we considered 2 repositories of pathways and 2 algorithms for analysis. The 2 pathway databases used in this study to map genes to predefined pathways are (1) Kyoto Encyclopedia of Genes and Genomes (KEGG; Kanehisa Laboratories, Kyoto University, Kyoto, Japan), which provides a database of metabolic, regulatory, and disease pathways; and (2) MetaCore software (GeneGo, Inc, St Joseph, MI), which is a proprietary, manually curated database containing the probability of having the human protein-protein, protein-DNA and protein compound interactions, metabolic and signaling pathways, and the effects of bioactive molecules. KEGG contains approximately 250 canonical signaling and metabolic pathways; Metacore software contains approximately 450 such pathways.

The 2 approaches used to analyze the pathways were: (1) statistical analysis for overrepresentation[21] and (2) a novel impact analysis.[22] The first method assesses the probability of having the observed number of differentially expressed genes on a given pathway just by chance. A Fisher exact test was performed to evaluate these probabilities, using *R* (www.r-project.org). The impact analysis, performed with Pathway Express,[22] takes into consideration the number of differentially expressed genes on each pathway, the position of the genes within the pathway, and the signaling interactions between various genes as described by the pathway. Signaling interaction refers to the situation in which the change in the activity of a given gene affects the expression of another gene in a consistent way. Signaling pathways from KEGG define a number of signaling interactions that include, for example, activation, repression, inhibition, and phosphorylation. The exact definitions for all these types of signaling interactions can be found at http://www.genome.jp/kegg/. An impact value and probability value are assigned to each pathway. The probability values that were obtained from both analyses were adjusted with the use of the false discovery rate method[23]; a probability value of $<.05$ was considered statistically significant.

Network connectivity analysis

An additional analysis was carried out to evaluate the potential importance of individual nodes in protein interaction networks for providing connectivity among differentially expressed genes.

To identify such "topologically significant" proteins, the set of differentially expressed genes was used to construct the shortest path network that connected corresponding nodes in the global database of protein interactions (MetaCore software). Next, the number of all paths that traversed each node in this shortest path network that was specific for genes differentially expressed in labor was computed. For each node, this number was compared with the total number of all paths going through the same node in the global network. With these numbers and the relative size of the differential gene set, probability values were calculated for each node in the shortest path network. A conservative Bonferroni correction was used to correct for multiple testing, and a probability value of $<.01$ was deemed significant. The probability value for a node indicates the likelihood that the node provides connectivity among the original set of differentially expressed genes. The nodes that are deemed significant by this method are displayed on pathways maps, where their functional roles can be evaluated further. A detailed description of the connectivity analysis is available as supplementary material on the web site of the Journal.

Real time quantitative real-time reverse transcriptase polymerase chain reaction assays

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) assays of selected genes were performed on a set of cervical biopsy samples different from those used in the microarray analysis. Patients who were included were those who underwent elective cesarean section with an unripe cervix (not in labor at term) and patients after spontaneous vaginal delivery (term labor). A detailed description of the methods and analysis is available as supplementary material on the web site of the Journal.

Results

Pathway analysis

Over-representation method on signaling and metabolic pathways. Fifty-two of the 450 pathways in the MetaCore database were significant based on the overrepresentation analysis ($P < .05$). The top 20 of these are listed in Table 1; the entire list can be found as supplementary material on the web site of the Journal. The 5 most significant pathways were (1) chemokines and adhesion, (2) extracellular matrix remodeling, (3) plasmin signaling, (4) plasminogen activator, urokinase (PLAU) signaling (Figure 1), and (5) vascular endothelial growth factor (VEGF)–family signaling. The figures of the remaining pathways can be found as supplementary material on the web site of the Journal.

The overrepresentation analysis performed on the KEGG pathways identified, 4 significant pathways were: cytokine-cytokine receptor interaction, complement and coagulation cascades, calcium signaling pathway, and arginine and proline metabolism (Table 2).

Impact analysis on KEGG signaling pathways. Impact analysis restricted to KEGG signaling pathways that were performed with Pathway Express identified 7 significant pathways, among which there were 2 pathways that were not significant according to the over-representation method: leukocyte transendothelial migration and epithelial cell signaling (Table 3).

TABLE 2
Significant pathways*

Map name	Genes in reference array (n)	Genes in differentially expressed list (n)	Adjusted *P* value
Metacore			
Chemokines and adhesion	206	20	3.85E-06
Extracellular matrix remodeling	60	12	3.85E-06
Plasmin signaling	47	10	1.42E-05
PLAU signaling	46	9	6.64E-05
VEGF-family signaling	45	9	6.64E-05
KEGG			
Cytokine-cytokine interaction	519	44	2.33E-08
Complement and coagulation	139	17	2.76E-05
Calcium signaling pathway	600	6	.0392
Arginine and proline	110	9	.0493

* Number of genes in the array = approximately 19,886.

Plasmin/PLAU signaling pathways. The plasmin and PLAU signaling pathways were among the most highly significant. Further investigation with the MetaCore database identified multiple transcription factors that activate the expression of plasminogen activator, tissue-type (PLAT): JunB, JunD, FOSL2, FosB, CREM, and c-FOS (Figure 2).

The PLAU signaling pathway (Figure 1) displays plasminogen activator urokinase binding to its receptor on the cell surface, subsequent binding to JAK1, and phosphorylation of STAT1 that leads to activation. The pathway is truncated at this point in the MetaCore pathway database. Thus, the relationship between activation of STAT1 and the differentially regulated genes in the uterine cervix before and after labor was explored by network analysis. Network analysis indicated that activation of STAT1 is linked to regulation in expression of several genes that are listed on the right side of Figure 3. JAK1 provides an essential network conduit between plasminogen activator urokinase receptor and several differentially expressed targets of STAT1.

Connectivity Analysis of MetaCore Pathways. Multiple nodes within the plasmin and PLAU signaling pathways were found to be topologically significant on the basis of their function of providing connectivity (Figure 1).

qRT-PCR

qRT-PCR assays were performed to measure messenger RNA levels of selected genes that are involved in the plasmin and PLAU signaling pathways. PLAU receptor (PLAUR), plasminogen activator inhibitor type 1 (SERPINE2), serine protease inhibitor (LEKT1), and fibroblast growth factor 2 (FGF2) were selected for further study. When we examined the top 5 pathways, FGF2 and LEKT1 were found to be specific to the plasmin and/or PLAU signaling pathways.

Consistent with the microarray analysis, qRT-PCR revealed FGF2, PLAUR, and SERPINE2 to be significantly upregulated in term labor patients, when compared with term no labor. LEKT1 was significantly downregulated after term labor (Figure 4).

Comment

Principle findings of this study are: 1) cervical dilation and remodeling after term labor is associated with specific gene signature networks; 2) fifty-two Metacore database metabolic and signal-

TABLE 3
Pathway Express, ranked by impact factor

Pathway name	Corrected *P* value
Cell adhesion molecules	2.74E-14
Cytokine-cytokine receptor interaction	8.15E-09
Signaling system	1.87E-06
Complement and coagulation cascades	.0145
Leukocyte transendothelial migration	.0176
Focal adhesion	.0235
Epithelial cell signaling related to specific infections	.0449

ing pathways involve the activity of the genes of the cervical transcriptome of spontaneous term labor; 3) the 5 most significant of these pathways were chemokines and adhesion, extracellular matrix remodeling, plasmin signaling, PLAU signaling, and VEGF-family signaling; 4) a network analysis identified multiple transcription factors that activate the expression of PLAT; 5) the same network analysis also indicated that JAK1 provides an essential network conduit between PLAUR and several differentially expressed targets of STAT1; and 6) qRT-PCR confirmed the involvement of the plasminogen/plasmin system in the process of cervical dilation and remodeling after labor.

The use of pathway analysis to derive gene "signature networks" allows for the following: 1) the ability to transition from biology at the molecular level to a more global systems approach to disease/biological processes; 2) the identification of key regulators or transcription factors that may not have been identified by microarray analysis; and 3) further interpretation of gene expression data by providing information on the protein-protein interaction, metabolic, signaling, and transcription-regulatory networks.[17,24-26]

It has been proposed that cervical changes during pregnancy occur in 4 phases: softening, ripening, dilation, and changes that occur after parturition.[27] We present a unique report of the signaling and metabolic pathways that are involved in phase 4 of this process: cervical dilation and remodeling after term labor. In the current study, 52 pathways that are documented by the Metacore database were noted to be significant. Some of them represent canonic pathways that can be found also in KEGG, although others are proprietary. The use of novel pathway analysis methods (Impact Factor [Pathway Express])[22] and Connectivity [Metacore]) confirmed the involvement of several of these signaling pathways and genes that lead to a consistent result of what represents the processes that are involved in cervical dilation and remodeling after term spontaneous labor.

Several pathways that were found to be significant (chemokine and adhesion, extracellular matrix remodeling, cytokine-cytokine interaction, VEGF-family signaling) contain genes that have been previously suspected as involved in cervical dilation and remodeling.[1-8,10-14,27-30] Different database and pathway analysis methods confirmed these findings.

Of interest was the finding that the complement and coagulation cascades (determined by Pathway Express), which include portions of the PLAU signaling and plasmin signaling pathways (determined by Metacore), are involved in cervical dilation and remodeling after labor. The plasminogen activation cascade is a proteolytic enzyme system that converts plasminogen to the active serine protease plasmin. Plasmin degrades most extracellular matrix proteins.[31] Plasminogen activation plays a role in the remodeling of the extracellu-

FIGURE 2
Network of transcription factors that activate the expression of PLAT

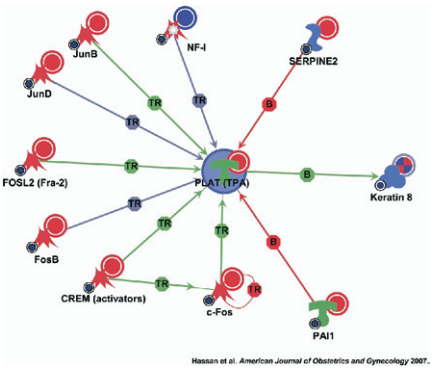

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FIGURE 3
JAK1 provides essential network conduit between PLAUR and many differentially expressed targets of STAT1

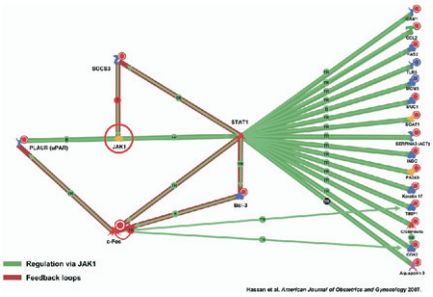

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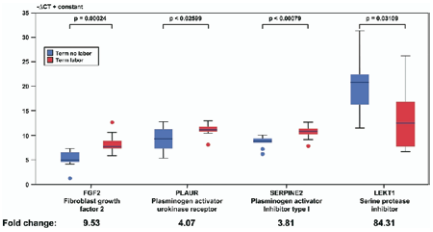
FIGURE 4
qRT-PCR results for FGF2, PLAUR, SERPINE2, and LEKT1



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 which is no >1.5 times the interquartile range from the box.

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lar matrix in human amnion, choriodecidua, and placenta during and after labor.[32] In addition, plasminogen activator inhibitor activity is increased in the serum of pregnant women near term, when compared with nonpregnant women and decreased after delivery.[33] Degradation of the cervical extracellular matrix may occur as a result of the activation of the plasminogen system. The finding of upregulation of multiple transcription factors that are involved in the activation of PLAT provides further support for the involvement of this pathway in cervical dilation and remodeling after term labor.

It is noteworthy that the macrophage migration inhibiting factor (MIF) in innate immune response pathway was significant. MIF encodes a lymphokine that is involved in cell-mediated immunity, immunoregulation, and inflammation. It plays a role in the regulation of the macrophage function in host defense through the suppression of antiinflammatory effects of glucocorticoids.[34] Elevated amniotic fluid concentrations of MIF are associated with intraamniotic inflammation, histologic chorioamnionitis, and shorter amniocentesis-to-delivery interval in patients in preterm labor.[35]

Interestingly, this study has identified Shc and GRB2 as significant nodes. Both are important mediators in MAPK-related signal transduction. Both mediate response to various growth factors and inflammatory response. The involvement of MAPK cascade during parturition has been demonstrated recently.[36] The role of these pathways in the mechanisms that are involved in cervical dilation and remodeling in term labor require further investigation.

The understanding of the gene signature pathways before and after labor in the uterine cervix is central to the molecular elucidation of the mechanisms responsible for cervical insufficiency, preterm labor, and arrest of dilation. Thus far, these common complications of pregnancy have eluded pathophysiologic definition.

A major strength of this study is that this report represents the first pathway analysis of the uterine cervix before and after human term labor and delivery. In addition, several significant pathways were noted to be consistently important when evaluated by several methods. The results of these analyses are consistent with previous results. However, we also highlight pathways that had not been implicated previously. The report of the plasminogen activation cascade and the pathways of innate immunity playing substantial roles in cervical dilation and remodeling is novel. Changes in the expression of several genes involved in some of the reported pathways were confirmed by qRT-PCR.

A limitation of this investigation is that we were unable to follow temporally the changes that are seen in an individual's cervix as labor progresses because of the obvious constraints of human research. Of note, the 2 patient groups differ in their obstetric history. Although it is not certain that this could account for any changes that are seen in gene expression, we wish to point out this difference.

Other types of pathway analyses have been previously used to examine the changes in myometrium in animal models. Salomonis et al[37] examined the mouse myometrium in the nonpregnant, mid gestation, late gestation, and postpartum states and defined gene expression changes under these conditions. Hierarchical-ordered partitioning and collapsing hybrid and GenMAPP 2.0 pathway (Gladstone Institutes, University of California at San Francisco, CA) analysis identified term quiescence, term activation, and postpartum involution expression patterns. There are no previous reports of the mechanisms that are involved in cervical dilation and remodeling after spontaneous labor and delivery with the use of pathway analysis.

The processes of cervical dilation and remodeling are the result of the activation of several pathways that have been implicated in the common terminal pathway of parturition. The pathways that are involved in this complex process include networks that are involved in chemokine and adhesion activation, cytokine-cytokine activity, extracellular matrix remodeling, the plasminogen system, recognition by the innate immune system, and the complement and coagulation cascade.

This investigation provides a unique global view of the changes that are seen in the uterine cervix after term labor and delivery. Remaining unanswered questions include the timing of the activation of each cascade, the role of each pathway in patients with cervical disease that result in abnormal term (arrest of dilation) and preterm birth (preterm labor, cervical insufficiency), and effective treatment strategies for cervical disease in pregnancy. The understanding of the pathways that lead to the changes in the cervix during labor and delivery may be critical to unraveling the solutions for the treatment of cervical disease in pregnancy. Future investigation of effective treatments for cervical disease in pregnancy should be targeted to the processes that have been identified as playing a critical role in the metabolic and signaling pathways that have been identified. In addition, future studies should focus on the remaining 3 components of system-level understanding: system dynamics, control, and design. ■

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