Gene Expression Profiling of Neonatal Mouse Uterine Development

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ABSTRACT

Postnatal uterine development involves differentiation and development of the endometrial glandular epithelium from the luminal epithelium as well as development of the mesenchyme into the endometrial stroma and myometrium. This period of development is critical because exposure of neonates to endocrine disruptors compromises reproductive cycles and pregnancy in the adult. However, the hormonal, cellular, and molecular mechanisms regulating postnatal uterine development remain largely unknown. In order to identify candidate genes and gene networks that regulate postnatal uterine development, uteri were collected from CD-1 outbred mice on postnatal days (PND) 3, 6, 9, 12, and 15, and gene expression profiling was conducted using Affymetrix mouse genome U74Av2 GeneChips in study 1. Of the approximately 12000 genes analyzed, 9002 genes were expressed in the uterus and expression of 3012 genes increased or decreased 2-fold during uterine development. In study 2, the uterine epithelium was enzymatically separated from the stroma/myometrium on PNDs 3, 6, and 9, and gene expression profiling was conducted using CodeLink UniSet Mouse 1 Expression Bioarrays. Results from these two studies support the hypothesis that postnatal uterine development is a complex process involving overlapping positive and negative changes in uterine epithelial and stromal/myometrial gene expression. Candidate genes regulating uterine development encode secreted factors (Wnt5a, Wnt7a), transcription factors (Hoxa10, Hoxa11, Hoxd10, MSX-1), enzymes (matrix metalloproteases, cathepsin, carbonic anhydrase), growth factors (IGF-II, IGF binding proteins), and components of the extracellular matrix (osteopontin) to name a few. The candidate genes and gene networks identified by transcriptional profiling provide an important foundation to discern and understand mechanisms regulating postnatal uterine morphogenesis.

developmental biology, female reproductive tract, gene regulation, uterus

INTRODUCTION

The mature uterine wall in the adult is comprised of two functional compartments, the endometrium and myometrium [1]. The endometrium is the inner mucosal lining of the uterus, derived from the inner layer of ductal mesenchyme [2, 3]. Histologically, the mouse endometrium consists of two epithelial cell types, luminal epithelium (LE) and glandular epithelium (GE), and stroma as well as blood vessels and immune cells. The myometrium is the smooth muscle component of the uterine wall that includes an inner circular layer, derived from the intermediate layer of ductal mesenchymal cells, and an outer longitudinal layer, derived from subperimetrial mesenchyme. During early fetal life, the uterus develops initially as a specialization of the mesodermally driven paramesonephric or Müllerian ducts, which is also the progenitor of the oviducts, cervix, and the entire upper vagina [1]. Although uterine histogenesis begins in utero, it is not completed until after birth, with the differentiation and development of the endometrial GE from the LE as well as the inner circular and outer longitudinal layers of the myometrium from the uterine mesenchyme in the neonate [4, 5]. Interestingly, endometrial gland morphogenesis, also termed adenogenesis, is predominantly a postnatal process in humans as well as in domestic and laboratory animals [5]. The endocrinological, cellular, and molecular mechanisms regulating postnatal uterine development are largely unknown in all mammals.

In the fetus, patterning and specification of the Müllerian duct involves Wnt7a, Hoxa10, and Hoxa11 [6–8]. Although Wnt7a null mice lack endometrial glands in the uterus of the adult [8], it is not clear if the uterus is properly specified to be uterus due to global posterior shift in development of reproductive tract of Wnt7a null mouse [6–8]. Ovarian and adrenal steroids do not influence uterine development in rodents before puberty [9–11]. Epithelial-mesenchymal interactions are recognized to play important roles in postnatal development of the uterus [2]. For example, the epithelium of the uterine endometrium plays an important promotional role in the differentiation of the myometrium in the postnatal mouse [3, 12]. However, these developmentally important regulatory mechanisms are still not well understood.

Perinatal uterine development represents a critical morphogenetic period because exposure to endocrine disruptors can have long-term negative consequences for adult uterine function and reproductive health [13]. Indeed, the success of uterine development in the neonate ultimately dictates the embryotrophic potential and function of the adult uterus [4, 5, 14–16]. Given the current lack of mechanistic knowledge, the systematic elucidation of uterine gene expression patterns during postnatal development is expected to provide a basis for understanding this complex process at the cellular and molecular levels. The overall hypothesis is that perinatal uterine development is complex, involving multiple gene networks and regulatory pathways. As a first step in testing this hypothesis, studies were conducted to 1) profile global changes in gene expression in the mouse uterus on postnatal days (PNDs) 3, 6, 9, 12, and 15 and 2) identify genes predominantly expressed in the uterine epithelium compared with stroma/myometrium in neonatal mice on PNDs 3, 6, and 9.
**MATERIALS AND METHODS**

**Animals and Tissue Collection**

The University Laboratory Animal Care and Use Committee of Texas A&M University approved all animal experimental procedures. Adult virgin CD-1 female mice were obtained from Charles River Laboratories (Wilmington, MA) and mated with fertile males of the same strain to establish pregnancy. Day of birth was designated as PND 0. All mice were housed in a temperature-controlled room (~21–25°C) under a 12L:12D cycle in the Kleberg Center Animal Facility (Texas A&M University) and provided fresh reverse osmosis/deionized water and NIH-31 lab chow ad libitum.

**Study 1.** For histology and global transcriptional profiling of the whole developing uterus, uteri were obtained from at least two different litters of mice on each PND 3, 6, 9, 12, or 15 (n = 15 mice per PND). Any remaining oviduct and cervix tissue was carefully removed from the uterus. The entire uterus was then snap frozen in liquid nitrogen and stored at −80°C for RNA extraction. In some cases, the midportion of each uterine horn was fixed in 4% paraformaldehyde in PBS (pH 7.2) at room temperature for 24 h. The fixed uteri were then processed and embedded in Paraplast Plus (Sherwood Medical, St. Louis, MO), sectioned at 5 μm, and stained with hematoxylin and eosin for morphological assessment.

**Study 2.** For identifying genes predominantly expressed in the endometrial epithelium as compared with the stroma/myometrium of the developing postnatal mouse uterus, the epithelium was isolated from the uteri using methods described previously [17]. Briefly, uteri were obtained from mice on PNDs 3, 6, or 9 (n = 10 mice per PND). The uterus was placed in small beakers and digested with 1% trypsin in Hank balanced salt solution for 60 min at 4°C. After brief DNase treatment, the uterine epithelial cells were separated from the stroma/myometrium using a glass pipette. The separated cell types were then snap frozen in liquid nitrogen and stored at −80°C for RNA extraction. Preliminary histological and gene expression analyses found that this method was not successful for isolating pure populations of uterine stroma/myometrium after PND 9 due to the extensive development of endometrial glands in the lower stroma (data not shown).

**Microarray Analysis**

**RNA isolation.** Frozen uteri (study 1) or isolated uterine cell types (study 2) were thawed in TRIZol reagent (Life Technologies, Gaithersburg, MD), and total RNA was isolated according to the manufacturer’s recommendations. The quantity and quality of total RNA was determined using Agilent Technologies 2100 Bioanalyzer (Palo Alto, CA) and by determining agarse gel electrophoresis. For study 2, purity of separated RNA was assessed by reverse transcription-polymerase chain reaction (RT-PCR) using calbindin-D28k for epithelium and smooth muscle alpha-actin for mesenchyme using methods described below.

**Affymetrix array systems.** Experimental procedures, including synthesis of double-stranded cDNA and biotin-labeled cDNA target, were performed by the Texas A&M University Microarray Core Facility in the Laboratory for Functional Genomics according to the recommendations in the Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix, Inc., Santa Clara, CA). Briefly, double-stranded cDNA was synthesized from 15–20 μg of total RNA isolated from whole uteri (Study 1). The purified double-stranded cDNA was used to synthesize biotin-labeled cDNA targets for hybridization of a GeneChip Array using BioArray HighYield RNA Transcript labeling Kit (Catalog #900182; Affymetrix, Inc.). Purified labeled cRNA (20–30 μg) then was fragmented to the size of 35–200 base pairs. Before hybridization onto GeneChip Array, the quality of labeling and fragmentation was checked by agarose gel electrophoresis. Fragmented biotinylated cRNA (15 μg) synthesized separately from uteri on each PND was hybridized to three separate MG_U74Av2 Affymetrix GeneChip Arrays (n = 15 total arrays). After washing, the chips were scanned using an Agilent GeneArray Scanner (Affymetrix, Inc.) at the excitation wavelength of 570 nm. After scanning, each image was checked for major chip defects or abnormalities in hybridization signal as a quality control. Based on a proprietary algorithm developed by Affymetrix, analysis of hybridization signal intensity over the results for each gene determined if genes were present, marginal, or absent in the dataset. Genes that were deemed absent at all time points in the experiment were eliminated from further analysis.

Expression values were globally normalized to a preset value and analyzed using Affymetrix Microarray Suit Software 5.0 version for absolute and pairwise comparison analyses. Values for the mean and standard deviation of three replicate average difference scores was calculated for each gene. The raw data were further analyzed, and normalized expression levels for Affymetrix GeneChip data were generated using GeneSpring Version 5.0 software (Silicon Genetics, Redwood, CA). K-means gene clustering by Pearson correlation and further screening was also performed using GeneSpring software using previously described visual methods [18]. The significance level was set at P < 0.05.

**CodeLink bioarray system.** Experimental procedures, including synthesis of double-stranded cDNA and biotin-labeled cRNA target, were performed by the Genomics and Bioinformatics Facility Core in the NIEHS Center for Environmental Health and Development at Texas A&M University using the CodeLink UniSet Mouse 1 Expression Bioarray, containing 10 014 gene probes. Briefly, cRNA synthesis was performed as per manufacturer’s instructions using 10 μg of total RNA from either isolated uterine epithelium or uterine stroma/myometrium (study 2). First-strand cDNA was generated using Superscript II reverse transcriptase and a T7 primer. Subsequently, second-strand cDNA was produced using Escherichia coli DNA polymerase I and RNase H. The resultant double-stranded cDNA was purified on a QiAquick column (Qiagen, Valencia, CA), and cRNA was generated via an in vitro transcription reaction using T7 RNA polymerase and biotin-11-UTP (Perkin Elmer, Boston, MA). The cRNA was purified on an RNeasy column (Qiagen), quantified by spectrometry, and 10 μg was then fragmented by heating at 94°C for 20 min in the presence of fragmentase. The fragmented cRNA was hybridized overnight at 37°C in hybridization buffer to a UniSet mouse I Bioarray (n = 2 arrays per cell type) in an Innova 4080 shaking incubator (New Brunswick Scientific, Edison, NJ) at 300 rpm. After hybridization, the arrays were washed in 0.7X TNT buffer (1X TNT: 0.1 M Tris-HCl, pH 7.6, 0.15 M NaCl, 0.05% Tween-20) at 46°C for 1 h followed by incubation with streptavidin-Alexa 647 (Molecular Probes, Eugene, OR) at room temperature for 30 min in the dark. Arrays were then washed in 1X TNT twice for 5 min each followed by a rinse in 0.05% Tween-20 in water. The slides were then dried by centrifugation and kept in the dark until scanning. Slides were scanned on an Axon GenePix Scanner (Arlington, TX) using CodeLink Expression Scanning Software, and images were analyzed using CodeLink Expression Analysis Software. Expression values were globally normalized to the median expression value of the whole array spots, the normalized expression data were analyzed using GeneSpring software.

**Real-time PCR**

Real-time PCR was performed using Applied Biosystems GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green method. Primer pairs for mouse genes were designed with Primer Express Version 1.5 software (Applied Biosystems) and checked for sequence homology against known genes using a BLAST search (Table 1). Complementary DNA was synthesized from 10 μg total cellular RNA isolated from different PNDs using methods described previously [19]. Newly synthesized cDNA (250 ng) was used for PCR reactions. To ensure specific amplification, various negative controls (i.e., water only, reaction without primers, and templates derived without reverse transcriptase) were included in the PCR reaction. To assess assay reproducibility, select RT reactions were performed in triplicate from different RNA pools extracted from different PNDs followed by real-time PCR. Values for cycle threshold (Ct), the point at which exponential amplification of the PCR products begins, were determined using the Applied Biosystems software. The Ct values were analyzed by least-squares analyses of variance (ANOVA) using the General Linear Models procedure of the Statistical Analysis System (Cary, NC). The model included the Ct value of cyclophilin as a covariate to correct for differences in cDNA input into PCR reactions. Orthogonal contrasts were used to determine effects of postnatal day on uterine gene expression. The significance level was set at P < 0.05.

Expression level for real-time PCR analyses were calculated using formula of 2−△△CT, where △△CT was the normalized Ct value obtained from PND 3 sample of each primer set and △CT was the normalized Ct value obtained from the other PNDs, as suggested by Applied Biosystems. The CDSAs of the target genes were also synthesized separately from different pools of uteri collected at different times and used for real-time PCR analysis to validate the assay and it also serves as a validation of the feasibility of using only cDNAs from one pool of RNA for the array analysis. After each run, the dissociation curve of each primer set was generated to confirm specific amplification. Although the RNA used for cDNA synthesis is not DNase treated, the quality of newly synthesized RNA was checked using Agilent Technologies 2100 Bioanalyzer for potential DNA contamination. Finally, all the PCR products were run on 2.0% agarose gel to confirm the presence of a single amplicon.
TABLE 1. Primers used for real-time RT-PCR analysis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession name</th>
<th>Number</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<td></td>
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<tr>
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</table>

In Situ Hybridization Analysis

Partial cDNAs of 10 genes, selected from those exhibiting at least a 5-fold change in expression, were cloned by RT-PCR using specific primers and methods described previously [20]. All the cDNAs were cloned into pCRII vector using the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced. In situ hybridization was conducted on uterine tissues using methods described previously [21]. Antisense and sense radiolabeled cRNA probes were generated using appropriate polymerases by in vitro transcription with [α-^35^S]-UTP. Transcripts protected from RNAse digestion were visualized by liquid emulsion autoradiography. Slides were stored at 4°C for 1–2 wk as judged from autoradiographs, developed in Kodak D-19 developer (Eastman Kodak, Rochester, NY), counterstained with hematoxylin for imaging.

RESULTS

Uterine Morphology

On PND 3, the mouse uterus contained no histologically discernible endometrial glands (Fig. 1). The endometrium consisted of a single thin layer of columnar luminal epithelium (LE) supported by stroma. The uterine lumen was slightly folded, and the inner circular and outer longitudinal layers of the myometrium were apparent. By PND 6, the endometrium contained areas of LE thickening that represented budding endometrial glandular epithelium (GE). Tubular and slightly coiled endometrial glands could be observed in PND 9 uteri. By PND 12, the tubular glands were more coiled and some reached from the uterine lumen to the inner circular myometrium. By PND 15, the endometrial glands were visibly coiled and developed to the myometrium. The myometrium appeared to be fully differentiated. The histoarchitecture of the uterus on PND 15 was comparable with that of mature adult mice.

Global Profiling of Gene Expression in the Neonatal Mouse Uterus (Study 1)

Using a total of 15 Affymetrix mouse genome U74A DNA microarrays, the uterine expression patterns of 12,000 known genes or expressed sequence tags (ESTs) were analyzed for PNDs 3, 6, 9, 12, and 15. Of the approximately 12,000 genes represented on the microarray, 9002 genes were present or marginally present in the uterus. Expression of 3012 genes (or approximately 30%) changed at least 2-fold between PND 3 and PND 15. Gene expression levels were compared between each consecutive postnatal day. As illustrated in Figure 2, expression of genes both increased and decreased during postnatal uterine development. Given the large number of genes that changed during postnatal development, tabular summaries of the individual genes found to be increased or decreased at least 5-fold between PND 3 and PND 15 are available at http://animalscience.tamu.edu/ansc/FACULTY/spencer.html.

Given that the endometrial glands begin to develop between PNDs 5 and 7, changes in gene expression around PND 6 may be critical for uterine gland development.


FIG. 2. Changes in gene activation and gene suppression in the uterus between PND 3 and PND 15. Comparisons of normalized expression value were done between consecutive PNDs, and those that showed an increase or decrease of at least 2-fold are depicted.
Therefore, the temporal pattern of gene expression was determined by K-means clustering using the Pearson correlation with genes displaying at least a 2-fold or greater change in expression compared with PND 6. Based on visual inspection, six clusters best described the temporal gene expression profile (Fig. 3). The clusters contained a total of 733 genes, and the individual genes found to be increased or decreased at least 5-fold either before or after PND 6 are available as tabular summaries at http://animalscience.tamu.edu/ansc/FACULTY/spencer.html. Within those clusters, the percentage of ESTs ranged from 39.8% in cluster 2 to 51.6% in cluster 1.

Verification of Microarray Data

To validate gene expression patterns assessed by microarray, the expression of 15 selected genes was determined in the uterus using two-step real-time RT-PCR. This analysis verified expression patterns of approximately 70% of the selected genes (10 of 15) (Fig. 4). Assay reproducibility was confirmed by performing RT reactions in triplicate from RNA extracted from different pools of uterus before the PCR for several of the selected genes (data not shown). The C_T value of cyclophilin was used as covariate in ANOVA because cyclophilin gene expression was not affected (P > 0.10) by postnatal day (data not shown).

Identification of Genes Expressed in Uterine Epithelium and Stroma/Myometrium (Study 2)

The epithelium and stroma/myometrium were enzymatically separated from the uterus of mice on PNDs 3, 6, and 9. The purity of the isolated cell types was confirmed by RT-PCR analyses using calbindin-D28k for epithelium and smooth muscle α-actin for stroma/myometrium (data not shown). Adequate separation of these uterine cell types on PNDs 12 and 15 was not possible as determined by RT-PCR analyses of calbindin-D28k and α-actin (data not shown).

Gene expression profiles in RNA isolated from the uterine epithelium or stroma/myometrium was conducted using CodeLink mouse Uniset I Bioarrays. For initial analysis, the data sets were filtered using present and marginal flags to exclude those gene that were not present in the uterus. The data was then analyzed to determine which genes were expressed predominantly in the uterine epithelium or in the stroma/myometrium by comparing the normalized expression values of each gene in the cell types from each PND. Genes predominantly expressed (with a 5-fold or greater difference in normalized expression values) in the uterine epithelium and in the uterine stroma/myometrium are each available in tabular summaries at http://animalscience.tamu.edu/ansc/FACULTY/spencer.html.

In Situ Hybridization

Expression of selected genes was determined in mouse uterus using in situ hybridization analyses (Fig. 5). Expression of calbindin-D28k, carbonic anhydrase two (CAR2), homeobox msh-like 1 (MSX-1), and Wnt7a mRNA was detected only in the endometrial LE and GE, whereas expression of MMP-2, Hoxa10, Hoxa11, and BMP2 mRNA was detected only in the endometrial stroma. As expected, smooth muscle α-actin was expressed predominantly in the myometrium.

DISCUSSION

In the present studies, the combination of gene profiling of the entire developing uterus with that of isolated uterine epithelium and stroma/myometrium was effectively utilized to identify a number of potential genes and gene networks regulating uterine development in the neonatal mouse. Results of microarray analyses and clustering gene profiles support the overall hypothesis that perinatal uterine development is complex and regulated by multiple genes and gene networks expressed in the uterine epithelium and/or stroma/myometrium. In the present study, differences in expression of selected genes from the microarray analyses were verified and confirmed using a combination of real-time PCR and in situ hybridization analyses. The majority (70%) of selected genes identified by DNA microarray analysis were verified and confirmed by real-time PCR. Overall, genes with low normalized expression levels from the DNA microarray analysis also had low expression levels by real-time PCR analyses, i.e., high C_T values, and were not consistently detected using in situ hybridization analyses of developing mouse uteri. This finding was not surprising given the lower sensitivity of in situ hybridization techniques as compared with DNA microarray and real-time PCR analyses. A complete analysis of the biological significance of results from the present studies is cer-
FIG. 4. Comparison of temporal changes in uterine gene expression as determined by DNA microarray or real-time PCR analysis.
tainly beyond the scope of this article. Therefore, the following discussion highlights selected genes and gene networks, identified in the present studies, that are involved in development of other epitheliomesenchymal organs and candidate regulators of neonatal mouse uterine development.

Bone morphogenetic protein 2 (BMP-2), a member of the transforming growth factor beta (TGFβ) family is important in development of various organs [22, 23], and BMP-2 expression increased 7-fold in the mouse uterus between PND 9 and PND 12 in association with endometrial gland development. BMP receptor 1B null mice are sterile due, at least partially, to insufficient uterine gland development [24], suggesting that BMP signaling regulates, in part, uterine gland morphogenesis. In contrast, the uteri of Wnt7a null mice completely lack uterine glands [8]. In accordance with published findings, Wnt7a was constitutively expressed in the uterine epithelium between PNDs 3 and 15, whereas Wnt5a was expressed in the stroma/myometrium along with Hoxa10, Hoxa11, and Hoxd10. It is possible that Wnt7a is upstream of the Hox genes that are responsible for specification and patterning of the paramesonephric duct during fetal urogenital tract development. Therefore, the lack of endometrial glands may be attributed to a disruption in primary reproductive tract patterning that occurs in the fetus. Nonetheless, the Wnt and Hox gene network is present in the neonatal uterus and may represent an important epithelial-stromal network regulating endometrial differentiation in the developing uterus [25].

Elegant studies support a primary role for epithelial-mesenchymal interactions in uterine epithelial and mesenchymal development [2, 3, 12]. In the neonatal rat and ovine uterus, insulin-like growth factor (IGF) system has been implicated as an epithelial-stromal system mediating uterine development [20, 26]. Null mutation of IGF-I in the mouse revealed an essential role in uterine development [27]. The coordinated temporal and spatial expression of IGF-I and its binding proteins (IGFBP-1, -3, -4, -5, and -6) was hypothesized to play important roles in perinatal rat uterine development [26]. Although IGF-II expression was not detected in the neonatal rat uterus [26], IGF-II expression was detected in the stroma/myometrium of the developing neonatal mouse uterus in the present study, similar to observations in the ovine uterus [20]. Similar to the rat, IGF binding proteins 4 (IGFBP-4) and 7 (IGFBP-7) were predominantly expressed in the uterine stroma/myometrium of the developing mouse uterus in the present study. Collectively, available results from the neonatal mouse and other model systems support the idea that the IGF system regulates uterine development.

The matrix metalloproteinase (MMP) system regulates development and morphogenesis of a number of reproductive organs, including the uterus and ovary [28]. Recently, disruption of the tissue inhibitor of metalloproteinase-1 (TIMP-1) gene was found to increase the size and number of endometrial glands in the neonatal mouse uterus [29], implicating the MMPs in postnatal uterine development. Results of the present studies also implicate the MMP system (MMP-2, MMP-7, and ADAMTS-1) in uterine development. The MMP system regulates tissue remodeling through changes in the components of the extracellular matrix (ECM). Based on results from gene profiling, a large number of ECM components are present in and change during development of the neonatal mouse uterus. For instance, osteopontin, a secreted component of the ECM [30], increased almost 7-fold in expression between PND 12 and 15. The mechanistic role of the ECM and ECM-modifying enzyme systems in uterine development remains largely unknown but are likely to be important for tissue remodeling and morphogenetic events in the uterus [28].

Results of the present study represent the first step to identify genes and gene networks involved in perinatal uterine development using global transcriptional profiling. Currently, knowledge of the hormonal, cellular, and molecular
mechanisms regulating this process is very limited. These studies support our working hypothesis that neonatal uterine development involves coordinated changes in expression of many known and novel genes encoding transcription factors, growth factors, enzymes, and receptors. Future efforts will be directed at determining the mechanistic roles of these genes and gene networks in neonatal uterine development, which is a critical period that determines the success of adult uterine function.

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