Gene expression during successful implantation in a natural cycle

Inge Van Vaerenbergh, M.Sc.,[a] *Ramsey McIntire, Ph.D.,*[b] *Leentje Van Lommel, M.Sc.,*[c] *Paul Devroey, M.D., Ph.D.,*[d] *Linda Giudice, M.D., Ph.D., M.Sc.,*[b] *and Claire Bourgain, M.D., Ph.D.*[a]

[a] Department of Pathology, UZ Brussel and Vrije Universiteit Brussel, Jette, Belgium; [b] Department of Obstetrics, Gynecology and Reproductive Sciences, University of California San Francisco (UCSF), San Francisco, California; [c] Department of Molecular Cell Biology, Gene Expression Unit, KU Leuven, Leuven, Belgium; and [d] Centre for Reproductive Medicine, UZ Brussel, Jette, Belgium

Objective: To determine the human endometrial transcriptome during embryonic implantation.
Design: Case report.
Setting: Tertiary fertility center.
Patient(s): A 24-year-old woman who inadvertently became pregnant during an endometrial biopsy procedure.
Intervention(s): An endometrial biopsy was performed with a Pipelle device during the midluteal phase (days 19–21) of the cycle; blood samples for hormonal assessments were collected and a transvaginal ultrasound was performed.
Main Outcome Measure(s): Gene expression analysis of the endometrium during the window of implantation (during the implantation of an embryo) in a natural cycle. Localization of selected genes in endometrial tissue with immunohistochemistry.
Result(s): A total of 394 probe sets were differentially expressed in the pregnant sample when compared with the midsecretory phase nonpregnant endometrial samples. Different gene networks were involved, and selected genes from these signaling pathways were confirmed at the protein level.
Conclusion(s): Endometrial gene expression of a pregnant patient in a natural cycle is significantly different from nonpregnant patients during the midsecretory phase. (Fertil Steril® 2010;93:268.e15–e18. ©2010 by American Society for Reproductive Medicine.)

Key Words: Gene expression, implantation, pregnancy, endometrium, natural cycle

Cyclic uterine changes in the secretory phase have been described in detail in earlier studies by Armstrong et al. (1) and Gordon et al. (2). However, most observations on endometrial morphology were, and still are, performed in nonconception cycles. Reported series of endometrial biopsies in conception cycles are very limited. Although no adverse effect on implantation has been reported as a result of the endometrial biopsy procedure (3–5), an endometrial biopsy holds no morphological predictive information suggestive of ultimate pregnancy outcome (5, 6). Therefore, the aim of the current study was to define the endometrial transcriptome during the process of embryonic implantation.

CASE REPORT

This study was approved by the Ethics Committee of the University Hospital of the Vrije Universiteit Brussel.

As part of the assessment of the natural cycle, before a stimulated cycle in a controlled trial protocol, an endometrial biopsy (n = 1) was performed. The 24-year-old patient had a regular menstrual cycle and previously conceived twice in a natural cycle (G2P0A2). She did not suffer from endometriosis, polycystic ovary syndrome (PCOS), or any other endometrial pathology. An IVF treatment was indicated due to male factor infertility. The endometrial biopsy was performed with a Pipelle device 6 days (±1 day) after the LH surge, observed using urinary LH kits that were provided to the patient at the screening visit (first visit). At the day of the biopsy procedure, a blood sample was collected for hormonal assessments (P, midluteal phase) and a transvaginal ultrasound

Received June 16, 2009; revised August 16, 2009; accepted August 24, 2009; published online October 31, 2009.
I.V.V. has nothing to disclose. R.M. has nothing to disclose. L.V.L. has nothing to disclose. P.D. has received grant support from Merck, Schering Plough, Ferring, IBSA, Besins, Anecova. L.G. is a consultant for Repros Therapeutics, Quest Diagnostics, EMD Serono, Biosante, Vantia Therapeutics, and Bayer Schering Pharma; and owns stock in Pfizer. C.B. has nothing to disclose.
Supported by grant AL405 from Research Foundation-Flanders (FWO), Belgium, and by National Institute of Child Health and Human Development/National Institutes of Health, Bethesda, MD, through a cooperative agreement U54HD055764-02 (LCG) as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research.
Presented at the 25th Annual Meeting of the European Society of Human Reproduction and Embryology, Amsterdam, The Netherlands, June 28–July 1, 2009.
Reprint requests: Inge Van Vaerenbergh, M.Sc., Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium (FAX: +32 2 477 48 09; E-mail: ivvaeren@vub.ac.be).

0015-0282/10/$36.00

doi:10.1016/j.fertnstert.2009.08.057

examination of the ovaries and uterus was performed. The endometrial biopsy was divided into two parts: [1] histologic analysis, with hematoxylin and eosin (H & E) staining and [2] RNA isolation and additional gene expression analysis. The endometrial biopsy was evaluated with conventional histologic Noyes criteria (7) of secretory transformation by a specialized pathologist, unaware of treatment conditions.

Gene expression was analyzed with the Affymetrix HGU133 Plus 2.0 microarray (Affymetrix, Santa Clara, CA). The sample was used in duplicate as a "technical replicate" to reduce interassay variability and enhance the robustness of the microarray data, and hybridized using identical methods by two different laboratories (Gene Expression Unit, KU Leuven, Belgium and UCSF, Gladstone Genomics Core, San Francisco, CA) (8). The RNA sample was reverse-transcribed with the SuperScript Choice System (Invitrogen, Carlsbad, CA) with oligo-dT primers containing a T7 RNA polymerase promoter site. The resulting complementary DNA (cDNA) was in vitro transcribed and labeled with biotin using the IVT labeling kit (Affymetrix, Santa Clara, CA), followed by fragmentation of the biotinylated cRNA. Next, the quality of this cRNA was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The fragmented cRNA was hybridized overnight to the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix), containing more than 54,000 probe sets. Subsequently, the arrays were washed and stained according to the protocol (Affymetrix Expression Analyses Technical Manual) and scanned on a 2-μm Affymetrix 3000 GeneScanner. Data were analyzed with GeneSpring GX 7.3 (Agilent Technologies, Palo Alto, CA). The "pregnant" sample (in duplicate) was compared with the nonpregnant midluteal endometria (n = 8) from natural cycles. The probe level intensities data were preprocessed using the RMA algorithm (robust multiarray average; GeneSpring, Agilent Technologies, Palo Alto, CA) for background adjustment, normalization, and log-transformation of the perfect match values (9). In addition, the data were normalized per gene to the median. Data were then further analyzed with Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com, Redwood City, CA) for network and pathway analysis, as previously described (10). Selected genes were confirmed with immunohistochemistry on the pregnant patient sample (n = 1) and on control midluteal nonpregnant patient samples (n = 7). Commercially available antibodies were used (Abcam plc, Cambridge, United Kingdom), in addition to appropriate positive and negative controls (11–16). Antigen retrieval was performed by heating in citrate buffer (pH 6.0; ScyTek, Logan, UT) for 10 minutes in a water bath. Slides were incubated overnight with the appropriate primary antibodies at 4°C in a humidified chamber, treated with a horseradish peroxidase-conjugated secondary antibody (EnVision+ Dual Link System-HRP, Dako, Glostrup, Denmark), treated with diaminobenzidine (DAB, 10 minutes; Dako), and counterstained with hematoxylin. Staining intensity was scored semiquantitatively from 0 (negative) to 3 (intense) on the whole tissue by a single observer.

Hormonal assessment 9 days after the endometrial biopsy procedure showed a β-hCG value of 2,151 IU/L. Seven days later, the β-hCG value had increased to 20,525 IU/L. The patient had an ongoing pregnancy and delivered a healthy baby. It was calculated afterward from hCG values that implantation had occurred on the day of the endometrial biopsy procedure. The endometrial morphology was dated as day 6 of the luteal phase.

Of 54,675 probe sets, 394 were significantly differentially expressed between pregnant (n = 2, technical replicate) and nonpregnant (n = 8) midluteal endometrium in natural cycles (Volcano plot with Bonferroni correction; fold change $\geq$2.0, $P<.05$). Three hundred ten probe sets were up-regulated in the pregnant patient and 84 probe sets were down-regulated.

In total, 30 networks were derived from the list of 394 differentially expressed probe sets.

The top networks (according to their relevance to the list of genes) (10) were involved in cancer, reproductive system disease, post-translational modification, cell-to-cell signaling, cellular movement, and hematologic system development and function. Oxidative phosphorylation, ephrin receptor signaling, neurotrophin/tyrosine kinase receptor (TRK) signaling, purine metabolism, and peroxisome proliferator-activated receptor (PPAR) signaling were the most significant canonical pathways involved in the endometrium of the pregnant patient.

Four different genes, which are involved in these pathways, were selected to confirm tissue localization at the protein level. Immunohistochemistry showed cytoplasmic-positive staining, predominantly in the glandular compartment, in the endometrial biopsy from the pregnant patient, and in midluteal biopsies from control nonpregnant patients (Fig. 1) for Janus activating kinase 2 (JAK2), platelet-derived growth factor-A (PDGFA), cAMP response element-binding protein 3 (CREB3), and mitogen-activated protein kinase kinase 1 (MEK1). The staining pattern in glands and stroma was comparable between the pregnant sample and nonpregnant samples, with stronger intensity in nonpregnant tissues for PDGFA (glands scored as 3 in nonpregnant sample, scored as 2 in pregnant sample) and MEK1 (glands scored as 3 in nonpregnant, scored as 2 in pregnant sample).

This is the first report to compare the gene expression of conceptional midluteal endometrium with that of nonconceptional midluteal endometrium. Gene expression showed a differential expression of 394 probe sets, in which 30 networks were involved. Four up-regulated genes, belonging to the ephrin signaling pathway (Fig. 1), were selected to confirm protein expression and tissue localization. These were differentially regulated in conception midluteal endometrium compared with nonconception midluteal endometrium on the RNA level (Fig. 1). Immunohistochemistry also showed the presence of the selected genes on the protein level in the pregnant patient, as well as in nonpregnant patients. However, this technique is neither quantitative nor sensitive enough to differentiate between the two groups (17). Confirmation with a more quantitative technique, like Western blot, would be