Uterine signaling at the emergence of the embryo from obligate diapause

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EMBRYONIC DIAPAUSE, the suspension of the embryo development at the blastocyst stage during the preimplantation period, leads to a delay of embryo implantation. In the American mink (Mustela vison), obligate embryonic diapause occurs each breeding season during reproductive life (10, 15). It averages from 2 to 3 wk but can be extended to more than 3 mo under specific experimental conditions (25). The chronology of the process is as follows: 6 days after the mating-induced ovulation and 4 days after fertilization in the oviduct, the embryo reaches the uterus (15). There, the embryonic cells enter a cell cycle arrest, presumably in G0/G1 phase, and undergo a reduction of their metabolism to a basal level, thus leading to the suspension of the blastocyst development (6). It is well established that termination of embryonic diapause in this species is induced by increasing secretion of prolactin from the pituitary gland (24). Prolactin induces ovarian luteal cell activation and an increase in progesterone secretion with consequences on the endometrium, resulting in blastocyst reactivation and subsequent implantation (10, 17, 27). Reciprocal embryo transfer experiments between mink and ferret, a closely related species with no embryonic diapause, indicated that embryonic diapause is maternally controlled and dependent on the maternal uterine environment (3). Moreover, increased activity of uterine protein secretion into uterine lumen has been associated with resumption of blastocyst development in mink (9). Together, those results support the hypothesis that termination of embryonic diapause is triggered by the uterine environment and secretion of uterine factors into the uterine lumen. Nonetheless, the nature of uterine signaling at emergence from embryonic diapause remains elusive.

The aim of the present study was to explore uterine modifications concomitant to blastocyst reactivation following embryonic diapause. A transcriptomic analysis was performed using the suppressive subtractive hybridization (SSH) technique to construct a library of genes expressed in the uterus specifically at blastocyst reactivation.

MATERIALS AND METHODS

Tissue collection. All procedures involving live animals were approved by the Comité de déontologie de la Faculté de Médecine Vétérinaire, Université de Montréal, which is accredited by the Canadian Council on Animal Care. Investigations were carried out over four successive annual breeding seasons from 2005 to 2009 using ranch mink of the Dark and Pastel varieties purchased from A. Richard (St. Damase, QC, Canada). During this period, mink females were bred to two fertile males according to usual commercial farming procedures. Uterine horns from females during diapause were collected 7–9 days after the final mating and prior to March 21 every year between 2005 and 2009 (Fig. 1A). Blastocyst reactivation was synchronized among females by a daily injection of 1 mg·kg⁻¹ day⁻¹ ovine prolactin (Sigma, Oakville, ON, Canada) beginning on March 21 and for the following 7 days (24). The first day of prolactin injection was designated day 1 after blastocyst reactivation. Unimplanted blastocysts were flushed from the uterus using TC-199 medium (Gibco, Burlington, ON, Canada) containing 10% vol/vol fetal bovine serum (Gibco). The observation of expansion of blastocysts following prolactin treatment confirmed that reactivation had occurred as described previously (6). For SSH analysis, uterine horns were collected during diapause (n = 12) and on days 3, 5, and 7 each of prolactin-induced reactivation (n = 4/stage) (Fig. 1A). For validation and to determine detailed spatiotemporal gene expression, uterine samples were collected during diapause and on days 1, 3, 5, 7, and 9 after prolactin-induced blastocyst reactivation (n = 3/stage). Uterine tissue samples were snap-frozen and conserved at −80°C for RNA extraction or fixed in paraformaldehyde at 4% wt/vol overnight and
kept at 4°C in phosphate-buffered saline (PBS) 1× until paraffin embedding for immunohistochemistry assays.

Total RNA extraction and mRNA isolation from uterine samples. Total RNA was extracted from whole uterine samples using the RNeasy Mini kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. Messenger RNA was isolated from total RNA using the Oligotex kit (Qiagen) as described by the manufacturer.

SSH. SSH was performed with the PCR-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA), as detailed in Ref. 18. This technique enables isolation of differentially expressed cDNA sequences between two pools of cDNAs, the tester (specific cDNAs of interest) and the driver (cDNAs of reference) (8). Messenger RNA from uterine samples collected on days 3, 5, and 7 after blastocyst reactivation was pooled and assigned to the tester group, whereas pooled mRNA from uterine samples collected during diapause was designed as the driver (Fig. 1B). In each group, 2 µg of mRNA was used for cDNA synthesis. Two subtractions were performed: (1) forward subtraction: uterine samples collected during reactivation (tester) vs. those collected during diapause (driver); and (2) reverse subtraction: uterine samples collected during diapause (tester) vs. those collected at reactivation (driver). The reverse subtraction is required to perform the differential screening validation (see below). Subtraction efficiency was assessed by determining the difference of cycle number required for detection of peptidylprolyl isomerase A (PPIA) amplification between subtracted cDNA and nonsubtracted cDNA by polymerase chain reaction (PCR). Primers used for PPIA amplification were 5’-TAAATGGCACTTG-TGGCAAGTC-3’ and 5’-CAGATGAAGAACTGGGAACCG-3’ (7).

Differential screening. Following SSH, subtracted cDNA fragments were submitted to a differential screening to remove false-positive cDNAs common to both the tester and driver samples in the forward subtraction. The forward subtracted cDNA fragments generated by the SSH technique were cloned into the pCRII cloning vector using the TA Cloning Kit Dual Promoter (Invitrogen, Carlsbad, CA) and transformed into Max Efficiency DH5α competent cells (Invitrogen), as described by Lefèvre and Murphy (18). Briefly, 1,000 clones were randomly selected, and the presence of the insert was confirmed by PCR for each clone using primer 1 (5’-TCGAGGCGCCGCGCCGGGCA GGT-3’) and primer 2 (5’-AGCGTGGTCGCGGCCGGCGCCGGGCA GGT-3’) (Clontech) according to the manufacturer’s instructions. Amplified cDNAs from positive insert-containing clones were spotted on eight identical Hybond-N+ membranes (Amersham Pharmacia Biotech, Baie d’Urfé, QC, Canada). Subtracted cDNA fragments from the forward and reverse subtraction as well as nonsubtracted cDNAs from the tester and driver were used as probes to hybrize identical dot blot membranes containing the amplified cDNAs from positive clones of the forward subtraction. Probes were 33P-labeled using (α-33P)-dCTP (PerkinElmer, Boston, MA). This procedure was performed in duplicate using the PCR-Select Subtraction Hybridization Screening kit (Clontech), following the manufacturer’s instructions. Hybridization signals on the membranes were digitized using a Storm 840 PhosphorImager, and densitometric quantification of the signals was carried out with the ImageQuant software (Pharmacia Biotech). Clones selected for sequencing were those showing ≥3-fold differences in signal intensity between forward and reverse libraries.

Sequencing and sequence analysis. Plasmid DNA was extracted from the positive bacterial clones from the forward subtraction and was sequenced using the SP6 universal primer with an Applied Biosystems 3730xl DNA analyzer (PE Applied Biosystems, Foster City, CA) at the McGill University and Genome Quebec Innovation Centre, Montreal, QC, Canada. Sequences were trimmed from vector and adaptor sequences using the National Centre for Biotechnology Information b2seq program (http://blast.ncbi.nlm.nih.gov/b2seq/ wblast2.cgi). Sequences were then compared against the nonredundant GenBank database using the online computer Basic Local Alignment Search Tool (BLASTn) software (1). Aligned sequences were considered as homologous when the percentage of similarity was >89%, the E value <1.0 e-11, and the length of similarity >100 nucleotides. All cDNA sequences identified in the current study were submitted to GenBank (accession nos. GH271800 to GH272100 and GH693995 to GH694029). For convenience, differentially expressed cDNA sequences homologous to known genes will be designed by the name of the corresponding homologous known gene. Identified gene products were compared against the Secretory Protein Database (4) to identify genes encoding for potential secreted proteins. Gene ontology, specifically biological processes associated with the genes that were found in the Secretory Protein Database, was investigated using the functional annotation categories based on the Gene Ontology Consortium annotation categories available in the Homo Sapiens Database for Annotation, Visualization, and Integrated Discovery bioinformatics software available online (DAVID; http://david.abcc.ncifcrf.gov/). Some differentially expressed genes could be classified in more than one category. Significance for overrepresented categories was defined according to the DAVID annotation system with a Fisher Exact P value <0.10.

Quantitative real-time PCR. One microgram of RNA was reverse transcribed with Superscript II Reverse Transcriptase (Invitrogen). The primers were designed based on the cloned cDNA sequences using the Primer Express software version 3.0 (PE Applied Biosystems; Table 1). Primers used to amplify the reference gene glyceraldehyde phosphodehydrogenase (GAPDH) were designed based on the mink GAPDH mRNA sequence (accession no. AF076283) and were 5’-TCCCCACCCCCCAATGGT-3′ and 5’-CCCTCCTGATGCTGTCA-3′. Primer concentrations were optimized according to the manufacturer’s recommendations, and specificity of amplified products was verified by dissociation curve analysis for each primer set. To evaluate PCR efficiency, standard curves were prepared for each of the identified genes and the reference gene using a pool of uterine cDNA from all samples collected during diapause and on days 3, 5,
Table 1. Primer sequences and concentrations used to validate the mink uterine differential expression pattern of 10 selected cDNA sequences homologous to known genes using quantitative real-time-PCR analysis

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequences</th>
<th>Concentrations, nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADIPOR1</td>
<td>Forward 5'-CGATCCATTTTATGAGACACTTTTT-3'</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTTTGAAAGGAGGCGACATT-3'</td>
<td>900</td>
</tr>
<tr>
<td>ALCAM</td>
<td>Forward 5'-CTTTGAAAGGAGGCGACATT-3'</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-GAGGTACTCCTTGAGATG-3'</td>
<td>900</td>
</tr>
<tr>
<td>AZIN1</td>
<td>Forward 5'-GAGGTACTCCTTGAGATG-3'</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-CTTTGAAAGGAGGCGACATT-3'</td>
<td>900</td>
</tr>
<tr>
<td>TXNL1</td>
<td>Forward 5'-CTCAAACAGATTCCACAGATGAC-3'</td>
<td>900</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'-TTTTTCTTTTCTCCTAA-3'</td>
<td>300</td>
</tr>
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</table>

ADIPOR1, adiponectin receptor 1; ALCAM, activated leukocyte cell adhesion molecule; AZIN1, antizyme inhibitor 1; GDF3, growth and differentiation factor 3; HMGN1, high-mobility group nucleosome binding domain 1; ODC1, ornithine decarboxylase; SAT1, spermidine/spermine N\textsuperscript{1}-acetyltransferase; SPARC, secreted protein, acidic, cysteine-rich; TGM2, transglutaminase 2; TXNL1, thioredoxin-like 1.

and 7 after embryo reactivation. PCR efficiency for each gene was coevaluated using LinRegPCR software. Triplicate real-time PCR amplifications were performed in a 10-μl reaction volume consisting of 1 μl of the appropriate concentration of both sense and antisense primers, 5 μl of Power SYBR Green PCR Master Mix (PE Applied Biosystems), 0.1 μl of Amperase uracil-N-glycosylase (PE Applied Biosystems), and 3 μl of cDNA diluted 1 in 15 in distilled water. Cycling conditions were 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Amplification, detection, and data analysis were performed with a 7500 Fast Real-Time PCR System (PE Applied Biosystems) and with the use of the Sequence Detection Software version 1.4 (PE Applied Biosystems). To measure the expression of the 10 selected genes during diapause and reactivation, quantification of gene expression was performed using the relative standard curve method as recommended by PE Applied BioSystems. Relative quantitation using the comparative threshold cycle (CT) method (ΔΔCT) was employed to quantify gene expression of high-mobility group nucleosome binding domain 1 (HMGN1) and secreted protein, acidic, cysteine-rich (SPARC) during diapause and on days 3, 5, 7, and 9 as described previously. Gene expression for each of three experimental samples for each stage was measured in triplicate.

Immunohistochemistry. Fixed and cross-sectioned uterine tissues were used to localize SPARC and HMGN1 protein expression during diapause and thereafter during embryo reactivation as described previously, with slight modifications (7). Retrieval of antigens was performed on deparaffinized and hydrated sections by 0.075% trypsin digestion at 37°C for 20 min. After a blocking step using 10% bovine serum albumin wt/vol at room temperature for 6 h in a humid chamber at room temperature, tissues were then incubated overnight at 4°C with 4 μg/ml of SPARC (H-14) or HMGN1 (HMG14; N-15) goat anti-human antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) in a dark and humid chamber. After washes in PBS 1× containing 0.1% vol/vol of Tween 20, sections were incubated with a biotinylated second antibody for 1 h at room temperature, and the Vectastain ABC kit (Vector Laboratories, Burlington, ON, Canada) with 3,3′-diaminobenzidine was used to detect the peroxidase activity. Negative control sections were submitted to the same procedures, except that the first antibody was replaced by 4 μg/ml of nonimmune goat serum.

Statistical analysis. Data from quantitative real-time PCR were analyzed using the JMP, version 7 (SAS Institute, Cary, NC) and Statistica 8.0 (StatSoft, Tulsa, OK). Wilcoxon rank sum test was employed to compare target gene expression during diapause and at reactivation. The effect of diapause and reactivation in each of days 3, 5, and 7 with the nonsubtracted library to detect differences was analyzed using Kruskal-Wallis ANOVA. Multiple mean comparisons of target gene mRNA between each day were undertaken using the multiple comparison of mean rank. Statistical differences were defined as P < 0.05 and tendencies as P < 0.1.

RESULTS

Construction and global analysis of the SSH library. The SSH was performed to enable construction of a library comprising differentially expressed uterine cDNA sequences between diapause and on days 3, 5, and 7 after reactivation. SSH efficiency was assessed by measuring the difference of PCR cycle numbers required for detection of PPIA amplification between subtracted cDNA and nonsubtracted cDNA (Fig. 2). Fifteen and five additional amplification cycles were necessary in the forward and reverse subtracted libraries, respectively, compared with the nonsubtracted library to detect PPIA amplification, thus reflecting a reduction of PPIA mRNA abundance after both subtractions. Following subtraction, differentially expressed cDNAs from the forward subtraction were cloned to construct the SSH library. Among randomly selected SSH clones (n = 1,000), 85.9% were single insert-containing clones that had an average length of 370 ± 82 (means ± SD) base pairs. Screening was undertaken to confirm the differential expression of the cDNAs generated by the SSH, resulting in identification of 45.6% (392 out of 859) of the cDNA clones that had a difference in hybridization signals of ≥3 between diapause and reactivated samples. Of these differentially expressed cDNA sequences from the mink uterus, 337 were successfully sequenced, and sequences were compared against the GenBank database. When considering the nonredundant list of differentially expressed genes, the uterine cDNA library
genes that were encoding for potentially secreted products and were represented by more than two clones. The two most sequenced. A total of 10 sequences homologous to known genes and/or implantation process. Three polyamine-related genes identified in the SSH library, ornithine decarboxylase 1 (ODC1), antizyme inhibitor 1 (AZIN1), and spermidine/spermine N\(_1\)-acetyltransferase (SAT1), encode for enzymes that are implicated in polyamine biosynthesis, which are secreted regulators of cell proliferation (29). Since polyamines play a role in rodent embryo implantation (11, 12, 31) and in vitro reactivation of mouse blastocyst following experimentally induced delayed implantation, ODC1, AZIN1, and SAT1 were also selected for SSH validation. As an adipokine receptor, adiponectin receptor 1 (ADIPOR1) has been proposed to be involved in human embryo implantation. Finally, we selected the HMGN1 gene, since it encodes for a chromatin remodeling factor that may regulate downstream expression of uterine gene associated with blastocyst reactivation. Comparison between mRNA abundance measured during diapause and mean mRNA abundance measured on days 3, 5, and 7 after reactivation was undertaken for those 10 selected genes (Fig. 3). The differential uterine gene expression pattern of those 10 genes was confirmed by mRNA abundance increased (\(P < 0.05\)) in uterine samples between diapause and blastocyst reactivation.

Given that our initial objective was to explore modifications of uterine environment associated with blastocyst reactivation comprised 211 unique sequences, among which 58% are homologous to known gene sequences (Supplemental Table S1; Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism website), 14% are homologous to known but uncharacterized sequences such as expressed sequenced tags, and 28% correspond to novel sequences. A total of 10 sequences homologous to known genes were represented by more than two clones. The two most redundant clones were \(\beta\)-defensin 139 (DEFB139; 56 clones) and tumor rejection antigen 1 (HSP90B1; 7 clones) (Supplemental Table S1).

Among the 123 identified genes, 41.5% were listed in the Secretory Protein Database (Supplemental Table S1). Functional annotation of gene ontology was enabled to determine overrepresented categories of biological processes among the genes that were encoding for potentially secreted products and differentially expressed in the uterus at embryo reactivation (Table 2). Among those genes, 14% that were implicated in the regulation of cell proliferation, 14% that were implicated in the regulation of cell homeostasis, 14% that were implicated in the regulation of homeostatic process, 11% that were implicated in the regulation of protein folding, 8% that were implicated in the regulation of electron chain, and 8% that were implicated in the regulation of innate immune system were significantly represented among differentially expressed genes at reactivation encoding for secreted proteins.

**Validation of SSH.** The differential expression pattern of 10 identified genes was investigated by quantitative (q)PCR (Table 1) to confirm SSH results. Those genes were selected according to the following rationale; thioredoxin-like 1 (TXNL1), transglutaminase 2 (TGM2), and SPARC represent overrepresented biological processes potentially involved in embryo reactivation (Table 2) and were previously referenced in the literature as being implicated in the preimplantation and/or implantation process. Activated leukocyte cell adhesion molecule (ALCAM) and growth and differentiation factor 3 (GDF3), also listed in the SSH library and Secretory Protein Database, were proposed to be involved in the preimplantation and/or implantation process. Three polyamine-related genes identified in the SSH library, ornithine decarboxylase 1 (ODC1), antizyme inhibitor 1 (AZIN1), and spermidine/spermine \(N_1\)-acetyltransferase (SAT1), encode for enzymes that are implicated in polyamine biosynthesis, which are secreted regulators of cell proliferation (29). Since polyamines play a role in rodent embryo implantation (11, 12, 31) and in vitro reactivation of mouse blastocyst following experimentally induced delayed implantation, ODC1, AZIN1, and SAT1 were also selected for SSH validation. As an adipokine receptor, adiponectin receptor 1 (ADIPOR1) has been proposed to be involved in human embryo implantation. Finally, we selected the HMGN1 gene, since it encodes for a chromatin remodeling factor that may regulate downstream expression of uterine gene associated with blastocyst reactivation. Comparison between mRNA abundance measured during diapause and mean mRNA abundance measured on days 3, 5, and 7 after reactivation was undertaken for those 10 selected genes (Fig. 3). The differential uterine gene expression pattern of those 10 genes was confirmed by mRNA abundance increased (\(P < 0.05\)) in uterine samples between diapause and blastocyst reactivation.

Given that our initial objective was to explore modifications of uterine environment associated with blastocyst reactivation

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**Table 2. Distribution of overrepresented biological process categories of the 51 differentially expressed genes in the mink uterus between diapause and reactivation listed in the Secretory Protein Database**

<table>
<thead>
<tr>
<th>GO Term</th>
<th>Genes</th>
<th>%</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0006457 ~ protein folding</td>
<td>HSP90B1, PPB1, PDA6, DNAJB1</td>
<td>11.11</td>
<td>0.01</td>
</tr>
<tr>
<td>GO:00019725 ~ cellular homeostasis</td>
<td>TXNL1, HSP90B1, TGM2, PDA6, CLN5</td>
<td>13.89</td>
<td>0.02</td>
</tr>
<tr>
<td>GO:00022900 ~ electron transport chain</td>
<td>TXNL1, SLC25A13, ND4</td>
<td>8.33</td>
<td>0.07</td>
</tr>
<tr>
<td>GO:0045087 ~ innate immune response</td>
<td>TMED7, C1R, DEFB139</td>
<td>8.33</td>
<td>0.04</td>
</tr>
<tr>
<td>GO:0042592 ~ homeostatic process</td>
<td>TXNL1, HSP90B1, TGM2, PDA6, CLN5</td>
<td>13.89</td>
<td>0.04</td>
</tr>
<tr>
<td>GO:0042127 ~ regulation of cell proliferation</td>
<td>PTGS1, TGM2, PTE, SPARC, CXADR</td>
<td>13.89</td>
<td>0.09</td>
</tr>
</tbody>
</table>

GO, gene ontology; SLC25A13, solute carrier family 25, member 13 (citrin); ND4; mitochondrially encoded NADH dehydrogenase 4; TMED7, transmembrane emp24 protein transport domain containing 7; C1R, complement component 1, r subcomponent; DEFB139, \(\beta\)eta-defensin 139; PTGS1, prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase); PTE, pleiotrophin; CXADR, coxsackie virus and adenovirus receptor. GO categories of biological processes and GO number, gene symbols of genes represented in each categories (in boldface are represented genes that were chosen for SSH validation), percentage of genes in each categories, and exact Fisher \(P\) values as defined by DAVID annotation system for significance of categories.
following embryonic diapause, we chose SPARC and HMGN1 for further evaluation. SPARC is a secreted glycoprotein known to be implicated in cell-cell adhesion (28) and cell proliferation (23) and was previously reported to be involved in mouse embryo implantation (30). Therefore, we proposed that SPARC and HMGN1 could reflect tissue and chromatin remodeling activity, respectively, of the uterus at blastocyst reactivation.

**Uterine expression of HMGN1 gene and protein at embryo reactivation.** The temporal expression pattern of HMGN1 was analyzed by qPCR over a wider temporal scale, beginning from diapause through day 9 after embryo reactivation in whole uterine samples (Fig. 4A). HMGN1 gene expression was elevated nearly twofold \((P < 0.05)\) on day 3 after embryo reactivation compared with diapause and tended to remain elevated thereafter on days 7 and 9 after reactivation after a slight decrease on day 5 after reactivation. To assess gene expression at the translational level and to evaluate cellular localization of HMGN1, protein immunolocalization in uterine cross sections was undertaken during diapause and on days 1, 3, 5, 7, and 9 after blastocyst reactivation. As seen in Fig. 4B, signal intensity of HMGN1 was strongly increased in mink uterine cross sections beginning on day 1 after reactivation compared with diapause. Indeed, the HG M N1 signal was only slightly detectable in the luminal and glandular epithelium, whereas it was more intensively present in the subepithelial stromal and glandular epithelial cells underlying the myometrium during diapause. Beginning 1 day after embryo reactivation, its expression was substantially increased in the luminal and glandular epithelial cells and in the subepithelial stromal cells throughout the endometrium. A similar distribution pattern for HMGN1 was observed on days 3, 7 (data not shown), and 9 after reactivation in the uterus (data not shown). On day 5 after reactivation, signal for HMGN1 exhibited a similar distribution, whereas intensity was reduced compared with that on days 1, 3, 7, and 9 after reactivation.

**Uterine expression of SPARC gene and protein at blastocyst reactivation.** Uterine abundance mRNA of the SPARC gene was increased significantly on day 3 after embryo reactivation compared with diapause and rose slowly until day 9 after reactivation after a slight but nonsignificant decrease on day 5 after reactivation (Fig. 5A). Immunolocalization of SPARC in the mink uterus at reactivation revealed an elevated expression of SPARC at the protein level as well (Fig. 5B). The presence of SPARC was detected in the luminal and glandular epithelial cells, and its concentration was locally higher at the apical pole of luminal epithelial cells during diapause. On day 1 after blastocyst reactivation, the intensity of the SPARC signal was increased in uterine epithelial cells and extended to subepithelial stroma cells throughout the endometrium. This specific distribution of SPARC could be observed on days 3, 7 (data not shown), and 9 after reactivation, whereas a reduction of SPARC signal intensity was detected on day 5 after reactivation.

**DISCUSSION**

The enigma of obligate embryonic diapause has fascinated researchers since the discovery of the phenomenon more than 100 years ago. The aim of the present study was to explore changes in the uterine environment associated with blastocyst reactivation following diapause. We performed a transcriptomic analysis of the mink uterus using the SSH technique, which successfully enabled the construction of a library that includes a total of 211 nonredundant and differentially expressed uterine cDNA sequences between diapause and embryo reactivation. This technique does not require previous knowledge of gene sequences and was consequently suitable for analyzing the mink genome for which a limited number of sequences are available in databases. In addition, the upregulation of gene expression between diapause and reactivation, measured by qPCR for 10 selected genes, demonstrated that the SSH was an efficient technique to generate differential expressed cDNA sequences in the context of the present study. Among the known and characterized or uncharacterized sequences, 78% were homologous to sequences found in the *Canis familiaris* database. This was expected, since both *Mus musculus* and *Canis familiaris* belong to the Caniformia monophyletic group of the Carnivora, and the dog genome is among the best characterized in this group (2). In addition, the SSH technique also generates unknown cDNA fragments or previously unknown genes, such as expressed sequence tags. Indeed, 31% of the nonredundant differentially expressed cDNAs corresponded to known but uncharacterized sequences and to unknown sequences. These unknown sequences were submitted to the GenBank database and will contribute to increase the number of the mink sequences available. They potentially correspond to novel genes that could be essential in the process of embryo reactivation and implantation following obligate embryonic diapause. Further analysis is underway to assess the functions of these novel genes in the context of the present study.

Among the genes identified, 41.5% were listed in the Secreted Protein Database, suggesting that they are encoding for products potentially subjected to secretion from the uterine...
epithelium into the uterine lumen. Increased uterine protein secretion into uterine lumen has been associated with resumption of embryo development in mink (9) and in skunk, which also presents embryonic diapause (20). The in vitro model developed by Moreau et al. (22) provided evidence of embryo reactivation only when mink embryos in diapause were cocultured with mink uterine cell monolayers. Our results are consistent with what was reported previously and reinforce the hypothesis that termination of embryonic diapause is triggered by uterine environment, namely secretion of specific uterine factors into the uterine lumen.

With respect to the Gene Ontology functional annotation of biological process categories, the genes encoding for potentially secreted products were distributed in the regulation of cell proliferation, homeostatic process, cellular homeostasis, protein folding, electron transport chain, and innate immune response categories. Thus, we proposed that secreted uterine factors induce blastocyst reactivation after diapause by regulating those biological processes in the blastocyst. Overrepresented categories of biological processes, such as the regulation of cell proliferation and protein folding, are consistent with previous studies that described a marked increase of embryonic protein and DNA synthesis within 72 h after initiation of reactivation, and embryo diameter increased thereafter in the mink (6). Our results also suggest that cellular homeostasis and electron transport chain may be regulated by uterine elements in the mink blastocyst at reactivation, a concept supported by increased mRNA abundance of TXNL1 and TGM2 at reactivation relative to diapause in the mink uterus. In vitro blastocyst reactivation following experimentally induced delayed implantation in mice was associated with an increase in blastocyst incorporation of amino acids. Amino acid transport systems are activated by a transmembrane electrochemical gradient resulting from modulation of electron transport chain of sodium ions that is increased in uterine fluids from delayed implantation mouse model after estrogen-induced blastocyst reactivation, and in vitro blastocyst reactivation was inhibited in low-sodium concentrated medium. Based on our data, we conclude that similar uterine-dependent mechanisms underlying regulation of cellular homeostasis and implicating activation of transport systems are involved in mink blastocyst reactivation.

The innate immune response was also a highly represented biological process at blastocyst reactivation in the mink uterus. Indeed, the most redundant clone found (56 redundant clones) in the SSH library contained a cDNA sequence homologous to the Canis familiaris/H9252-defensin 139 cDNA sequence. The /H9252-defensin family consists of antimicrobial peptides, which are
essential components of the innate immune response β-defensins that are produced according to a specific temporal pattern by the uterine luminal epithelium in women during menstrual cycles and pregnancy and act as a barrier to protect the uterus from infection that could compromise pregnancy (16). Thus, our data highlight an important increase of the innate immune response concomitantly with the rise of uterine secretion activity at reactivation. We propose that the later process has a protective role of the uterine and embryo environment from bacterial infections during the emergence of the blastocyst from diapause in mink.

The 10 selected genes that were chosen to validate the SSH are potential uterine factors for blastocyst reactivation after diapause in mink. Their differential expression pattern at reactivation measured in the present study generates new pathways to understanding of biological processes involved in uterine induction of blastocyst reactivation after diapause in mink. Further investigations on the implication of secreted uterine GDF3 and polyamines in mink blastocyst reactivation are now underway. The upregulation of HMGN1 and SPARC expression in the mink uterus at reactivation illustrates specific chromatin and tissue remodeling in the uterus associated with exit of the blastocyst from embryonic diapause. On day 5 after blastocyst reactivation, a slight decrease in HMGN1 and SPARC mRNA abundance and in the intensity of signals reflecting distribution of HMGN1 and SPARC protein in mink uterus could be observed consistently. Although no explanation for this observation is available, it may indicate that modifications of the uterine environment at blastocyst reactivation in mink follow orchestrated chronological events. HMGN1 encodes for a nonhistone nuclear protein that binds specifically to nucleosomes, promotes chromatin decondensation, and induces transcriptional activation of downstream genes (reviewed in Ref. 13). It was surprising not to localize HMGN1 in the nuclei of positive uterine cells, because it is a chromatin remodeling factor. Since HMGN1/nucleosome ratio varies from 0.01 to 0.1, the presence of nuclear HMGN1 protein may not be detectable in the present HMGN1 immunolocalization experiments. Cytoplasmic distribution of HMGN1 was reported previously and associated with mitosis, whereas HMGN1 nuclear localization was rather correlated with the interphase of the cell cycle (19), thus suggesting that uterine cell proliferation at mink embryo reactivation may be in part a HMGN1-dependent process during emergence from embryonic diapause. Further investigations are required to assess mechanisms and functions of HMGN1 in the mink uterus at embryo reactivation.
The present findings also revealed an increase in SPARC gene expression at the emergence from embryonic diapause in the mink uterus. Since SPARC encodes for a secreted glycoprotein belonging to the matricellular protein superfamily, modulators of cell-cell and cell-matrix interactions, it is involved in tissue remodeling (28). Its gene expression has previously been reported to be progesterone responsive in ovine uteri before embryo implantation (14), in uteri from cycling humans (5), and in cycling cattle (21) during the proliferative phase. Therefore, SPARC upregulation in the mink uterus at reactivation may result from reactivation of the corpus luteum and increased progesterone secretion associated with embryo reactivation (26). Since SPARC was described as an antiadhesive factor (23), SPARC may serve as an important uterine factor that prevents precocious mink embryo implantation during the period of embryo reactivation. The fact that apical poles of uterine luminal epithelial cells facing the uterine lumen were strongly stained for SPARC at reactivation supports that hypothesis.

To conclude, the present findings depict a transcriptomic view of the carnivore endometrium during blastocyst reactivation. This analysis revealed that 41.5% of the differentially expressed genes in the mink uterus at blastocyst reactivation encode for potentially secreted products. The gene ontology functional annotation of these genes demonstrated that regulation of cell proliferation, protein folding, and homeostasis may be biological processes orchestrated by the uterine environment and triggering emergence of the blastocyst from diapause. Our data also show that uterine innate immune response is activated at blastocyst reactivation, a critical period during which uterine environment and blastocyst are more vulnerable to bacterial infections. The present results also highlight substantial modifications in the uterus, such as chromatin and tissue remodeling, as exhibited by HMGN1 and SPARC elevated expression during that specific window of preimplantation embryo development in mink. Together, our results open new avenues on a better understanding of uterine signaling at the emergence of blastocyst from embryonic diapause.

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DISCLOSURES

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