Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes

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Abstract

The endometrium plays a central role among the reproductive tissues in the context of early embryo–maternal communication and pregnancy. It undergoes typical changes during the sexual/oestrous cycle, which are regulated by the ovarian hormones progesterone and oestrogen. To identify the underlying molecular mechanisms we have performed the first holistic screen of transcriptome changes in bovine intercaruncular endometrium at two stages of the cycle – end of day 0 (late oestrus, low progesterone) and day 12 (dioestrus, high progesterone). A combination of subtracted cDNA libraries and cDNA array hybridisation revealed 133 genes showing at least a 2-fold change of their mRNA abundance, 65 with higher levels at oestrus and 68 at dioestrus. Interestingly, genes were identified which showed differential expression between different uterine sections as well. The most prominent example was the *UTMP* (uterine milk protein) mRNA, which was markedly upregulated in the cranial part of the ipsilateral uterine horn at oestrus. A Gene Ontology classification of the genes with known function characterised the oestrus time by elevated expression of genes, for example related to cell adhesion, cell motility and extracellular matrix and the dioestrus time by higher expression of mRNAs encoding for a variety of enzymes and transport proteins, in particular ion channels. Searching in pathway databases and literature data-mining revealed physiological processes and signalling cascades, e.g. the transforming growth factor- β signalling pathway and retinoic acid signalling, which are potentially involved in the regulation of changes of the endometrium during the oestrous cycle.

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Introduction

During the sexual/oestrous cycle characteristic changes occur in the bovine endometrium regarding its composition and differentiation status. These changes are mainly regulated by the hormones progesterone (P_4), oestradiol and oxytocin (for review see Spencer *et al.* 2004). At oestrus, P_4 levels are low and increase after ovulation until the highest levels at dioestrus. The increase of P_4 is associated with the downregulation of its own receptor in the endometrial epithelium. Via oxytocin, luteolytic pulses of prostaglandin- $F_{2\alpha}$ are released leading to the regression of the ovarian corpus luteum, termed luteolysis (for review see Goff 2004). This results in the entry into a new ovarian cycle. In contrast, if an embryo is present and produces specific factors (e.g. interferon- τ) the luteolytic mechanism in the

endometrium is blocked and the functional corpus luteum is maintained, providing the basis for further development and the implantation of the conceptus. Although these basic means of hormonal regulations in the endometrium during the oestrous cycle are known, the detailed molecular mechanisms are not well understood.

In humans several studies using microarray analyses have been done investigating gene expression changes in the endometrium during the menstrual cycle. In two studies the proliferative phase was compared with the ‘window of implantation’ time (Kao *et al.* 2002, Borthwick *et al.* 2003) and in another two studies gene expression differences between the early secretory phase (2–4 days after the luteinising hormone (LH) surge) and the receptive phase (7–9 days after the LH surge) were investigated (Carson *et al.* 2002, Riesewijk *et al.* 2003).

Two recent studies also provided first insights into changes of gene expression in the endometrium during the oestrous cycle comparing the proliferative vs secretory phase in the mouse (Tan *et al.* 2003) and the Rhesus monkey (Ace & Okulicz 2004). For ruminants no comparable study has been done so far. Recently, we successfully applied a combination of subtracted cDNA libraries and cDNA array hybridisation to compare the mRNA expression profiles of bovine epithelial cells of the ipsilateral vs the contralateral oviduct (Bauersachs *et al.* 2003) and of cells from the ipsilateral oviduct at oestrus and dioestrus (Bauersachs *et al.* 2004) respectively. In the present study a similar approach was applied to analyse differential gene expression in the bovine intercaruncular endometrium at two important phases of the oestrous cycle – the time around ovulation (late oestrus) and the time of high P₄ (dioestrus) when the endometrium is prepared for communication with an embryo. Based on the results of bioinformatic analyses and literature data-mining, molecular pathways involved in the regulation of specific functions of the bovine endometrium during the oestrous cycle were identified.

Materials and methods

Synchronisation of oestrous cycle and collection of endometrial tissue samples

Six cyclic heifers (Deutsches Fleckvieh) between 18 and 24 months old were cycle synchronised by injecting i.m. a single dose of 500 µg cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at dioestrus. Animals were observed for sexual behaviour (i.e. toleration, sweating, vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate injection. All animals were checked by ultrasound-guided follicle monitoring starting 48 h after Estrumate application at intervals of 6 h. Blood samples were taken at day 20 and day 0 of the oestrous cycle every 6–9 h to determine serum LH levels (Schams & Karg 1969) and just before slaughtering to determine serum P₄ levels (Prakash *et al.* 1987). Three animals were slaughtered the morning after standing heat occurred within 8 h after the LH surge and three animals 12 days after oestrus; the former group displayed low serum P₄ levels (<1.0 ng/ml) and the latter had high serum P₄ levels (>6.0 ng/ml). The uterus was removed, opened longitudinally and divided into seven sections: corpus plus caudal, middle and cranial parts of the ipsilateral and the contralateral uterine horns (see Fig. 1a). Samples were carefully cut out from the lamina propria of the intercaruncular endometrium with a scalpel and immediately transferred into cryo-tubes and frozen in liquid nitrogen or on dry ice. Samples were stored at –80 °C until further processing. Tissue samples for *in situ* hybridisation were also taken from the same animals. All experiments with

animals were conducted with permission from the local veterinary authorities and in accordance with accepted standards of humane animal care.

Generation of subtracted cDNA libraries and cDNA array hybridisation

The production of subtracted libraries was done according to the suppression subtractive hybridisation (SSH) method (Diatchenko *et al.* 1996). Total RNA from endometrial tissue samples was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Equal amounts of RNA of all seven sections of the uterus were pooled for the preparation of subtracted cDNA libraries for oestrus and dioestrus. The construction of the libraries was done as previously described (Bauersachs *et al.* 2003, 2004). For every library, 1536 cDNA clones were randomly picked, the cDNA fragments amplified via PCR, spotted by a spotting robot onto small nylon membranes and analysed by array hybridisation (for details see Bauersachs *et al.* 2004). The cDNA arrays were hybridised with radioactively labelled probes derived from 42 tissue samples corresponding to the six animals. Up to 12 probes were hybridised in parallel. All hybridisations that were directly compared in the data analysis were processed simultaneously under equal conditions.

Analysis of array data

Array evaluation was done using AIDA Image Analyzer Software (Version 3.41; Raytest, Straubenhardt, Germany). Background was subtracted with the 'Weighted image regions' function. Raw data obtained by AIDA Array software were exported to Microsoft Excel and normalised to the mean signals of internal reference cDNAs of each array. Normalised data were compared pair-wise (oestrus vs dioestrus) using the datasets derived from the hybridisation experiments that were processed simultaneously. cDNA clones were set as differentially expressed between oestrus and dioestrus if they showed at least a 2-fold up- or downregulation in every oestrus–dioestrus pair in at least one section of the uterus. In Tables 1 and 2 the means of the ratios of the single uterine sections and the three pair-wise comparisons are shown. The ratios of the uterine sections were very similar for almost all genes. The coefficient of variation (CV) values for the three comparisons are also shown. If a gene was represented on the array by more than one cDNA fragment the mean expression difference was calculated. Differential expression across the uterine segments (uterine horn cranial to corpus) was analysed by ANOVA (parametric test, Benjamini & Hochberg false discovery rate) and a post-hoc test (Student–Newman–Keuls) using GeneSpring software

Version 6.1 (Silicon Genetics, Redwood City, CA, USA). The analysis was done separately for the two sides of the uterus and the two times of the cycle. The four values of every cDNA derived from the cranial, middle and caudal uterine horn, and the corpus over the mean of all four values were used to consider the relative changes between these four uterine sections.

Sequencing of cDNAs with differential hybridisation signals and data analysis

cDNA fragments showing differential hybridisation signals were sequenced directly from spotting solutions by automated DNA sequencing (3100-Avant Genetic Analyzer; Applied Biosystems, Langen, Germany). Resulting sequences were compared with public sequence databases using the basic local alignment search tool at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/blast.cgi). cDNAs without similar entries in the 'nr' database were in addition compared with the 'est' database or the raw version of the bovine genome (http://pre.ensembl.org/Multi/blastview?species=Bos_taurus). Based on the human homologues simplified GOs were built using the GeneSpring software. Resulting data were supplemented with additional information from LocusLink (www.ncbi.nlm.nih.gov/locusLink/) and Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene), and from the literature. Pathway analyses were done searching the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database (www.genome.ad.jp/kegg/pathway.html) and the literature (PubMed abstracts).

Real-time RT-PCR

The same 42 RNA samples as for array hybridisation were used. One microgram of each sample of total RNA was reverse transcribed in a total volume of 60 µl, containing 1 × buffer (Promega), 0.5 mM dNTPs (Roche), 2.5 µM hexamer primers (Gibco BRL, Grand Island, NY, USA) and 200 U Superscript RT enzyme (Promega). Primers were designed to amplify specific fragments referring to selected regulated genes: *CLDN10* (forward 5'-CATTTCATGCCAATCAGGG; reverse 5'-CGCTGGACGGTTACATCC (97 bp)), *CPN1* (forward 5'-AGAGAGGCCCTGCCTGAC; reverse 5'-AGAAAGAATGCTTCTTCTGGTG (90 bp)), *EPHX2* (forward 5'-ACTCCTTGTGAACACCCAG; reverse 5'-TAGAGGCCCTGAAACC (109 bp)), *INHBA* (forward 5'-CGATGGGCAGAACATCATC; reverse 5'-GTCTTCTTTGGACCGTCTCG (113 bp)), *MGP* (forward 5'-CCCGGAGACACCATGAAG; reverse 5'-GAGGGAGAGGGGAGAATCC (161 bp)), *MS4A8B* (forward 5'-CTGACCTGCTACCAAACCAAC; reverse 5'-GCCCAAACAAGGGAGGTATTC (191 bp)),

PENK (forward 5'-TCTGGAATGTGAGGGGAAAC; reverse 5'-CTTCTTAGCAAGCAGGTGGC (142 bp)), *PTHLH* (forward 5'-TCCCCTAACTCCAAGCCTGC; reverse 5'-TTTTCTTTTCTTGCCGGG (148 bp)), *SOX4* (forward 5'-ATTTCGAGTTCCCGGACTAC; reverse 5'-CCTTCAGTAGGTGAAGACCAGG (101 bp)), *UTMP* (forward 5'-ATATCATCTTCTCCCCCATGG; reverse 5'-GTGCACATCCAACAGTTTGG (126 bp)), and the housekeeping gene *Ubiquitin* (forward 5'-AGATCCAGGATAAGGAAGGCAT; reverse 5'-GCTCCACCTCCAGGGTGAT (198 bp)) (referring to Neuvians *et al.* 2003). All amplified PCR fragments were sequenced with forward and reverse primers (3100-Avant Genetic Analyzer; Applied Biosystems) to verify the resulting PCR product. Thereafter the specific melting point of the amplified product served as verification of the product identity (*CLDN10*, *CPN1* and *MS4A8B* 84 °C, *EPHX2*, *PTHLH* and *MGP* 85 °C, *INHBA*, *PENK* and *UTMP* 86 °C, *SOX4* 87 °C and *Ubiquitin* 88 °C) (Pfaffl *et al.* 2003). For each of the following real-time PCR reactions, 1 µl cDNA was used to amplify specific target genes. Quantitative real-time PCR reactions using the LightCycler DNA Master SYBR Green I protocol (Roche) were performed as described previously (Ulbrich *et al.* 2004). In each PCR reaction 17 ng/µl cDNA were introduced and amplified in a 10 µl reaction mixture (3 mM MgCl₂, 0.4 µM primer forward and reverse each, 1 × LightCycler DNA Master SYBR Green I; Roche) using a real-time LightCycler instrument (Roche). The annealing temperature was 60 °C for all reactions. To ensure an accurate quantification, a high temperature fluorescence measurement at 80 °C and 78 °C for *EPHX2*, *INHBA*, *MGP*, *PENK*, *PTHLH* and *UTMP* and *CPN1*, *CLDN10*, *MS4A8B* and *Ubiquitin* respectively, was undertaken in a fourth segment of the PCR. The cycle number required to achieve a definite SYBR Green fluorescence signal was calculated by the second derivative maximum method (crossing point, CP) (LightCycler software Version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration. As negative controls, water instead of cDNA was used.

Data analysis of real-time RT-PCR

The CPs determined for the target genes were normalised against the housekeeping gene *Ubiquitin*. Results are presented as means ($n=3$) (see Table 4). Expression ratios are presented as x-fold regulation between oestrus and dioestrus relating to each section. Differences between day 0 and day 12 for each section were analysed using one-way ANOVA. The normal distribution was tested by the Kolmogorow–Smirnov method, followed by a Student's *t*-test to find significant differences (Sigma-Stat, Version 2.03). The level of significance (*P*-value) is stated in Table 4.

In situ hybridisation

Formalin-fixed (3·7%), paraffin-embedded samples were used to localise transcripts of *PENK* (proenkephalin), *PTH1H* (parathyroid hormone-like hormone), *SAL1* (salivary lipocalin), *INHBA* (inhibin beta A) and *UTMP* (uterine milk protein precursor) within bovine uterus tissue (middle section of the ipsilateral horn). All samples except those used for detection of *PENK* mRNA were recovered during the oestrous phase. For *PENK*, material harvested at day 12 of the oestrous cycle was used. The same animals were used as for array hybridisation and real-time RT-PCR. Buffers (50 mM Tris-buffered saline (TBS) and 0·1 M sodium phosphate buffer) were adjusted to pH 7·4 unless otherwise noted. All solutions for *in situ* hybridisation were prepared using diethyl pyrocarbonate-treated water and glassware sterilised at 200 °C. In addition, all steps prior to and during hybridisation were conducted under RNase-free conditions.

Sections were deparaffinised with xylene (3 × 10 min), immersed in isopropanol (2 × 5 min) and then allowed to air dry. Dried sections were submerged in 2 × saline sodium citrate (SSC, pH 7·0) and pre-heated in a Bain-Marie water bath (80 °C) for 10 min followed by gradual cooling off for 20 min at room temperature. Slides were then washed in distilled water (2 × 5 min), TBS (2 × 5 min) and permeabilised for 20 min with 0·05% proteinase E (VWR, Ismaning, Germany) in TBS at room temperature. Sections were relocated in TBS (2 × 5 min) followed by distilled water (2 × 5 min) and post-fixed for 10 min in 4% paraformaldehyde/PBS (pH 7·4). After washing in PBS (2 × 5 min) and distilled water, slides were dehydrated in an ascending graded series of ethanol and air-dried. Hybridisation was carried out by overlaying the dried sections with 40 µl of the corresponding biotinylated oligonucleotide probe (100 pmol/µl), diluted 1:20 in *in situ* hybridisation solution (DAKO, Munich, Germany) and incubating them in a humidified chamber (using cover slips to prevent drying-out) at 38 °C overnight. RNase-free hybridisation solution (DAKO) contained 60% formamide, 5 × SSC, hybridisation accelerator, RNase inhibitor and blocking reagents. Subsequently, slides were washed in 2 × SSC (2 × 15 min, preheated to 38 °C), distilled water (2 × 5 min) and TBS (2 × 5 min). Detection of hybridised probes was performed using horseradish peroxidase-labelled ABC kit reagents developed with 3,3'-diaminobenzidine (DAKO) according to the manufacturer's instructions. Negative controls were done omitting the oligonucleotide probe and hybridisation with sense oligonucleotide probes. The sequences of the antisense oligonucleotides were as follows: *INHBA*: 5'-GATGATGTTCTGCCCATCG, *UTMP*: 5'-GTGCACATCCAACAGTTTGG, *PTH1H*:

5'-CAGGCTTGGAGTTAGGGGA, *SAL1*: 5'-GTATCTGTTCTCGGGCGT and *PENK*: 5'-GTTTCCCCTCACATTCCAGA.

Results

To identify differentially expressed genes in the bovine endometrium between the (late) oestrous and the dioestrous stage a combination of subtracted cDNA libraries and cDNA array hybridisation was applied. Two subtracted cDNA libraries were produced for oestrus and dioestrus respectively, starting from a pool of RNA samples derived from seven sections of the uterus (Fig. 1a). One thousand five hundred and thirty-six randomly picked cDNA clones of each library were analysed by cDNA array hybridisation with 42 samples of six animals (oestrus *n*=3, dioestrus *n*=3).

First, array hybridisation data were analysed for differences between oestrus and dioestrus. This analysis revealed 444 cDNA fragments showing at least 2-fold signal differences between oestrus and dioestrus in all three animal pairs (at least one uterine section). Figure 1b shows the hybridisation signals of one cDNA fragment of the *UTMP* (uterine milk protein) gene derived from the probes of one animal at oestrus and one at dioestrus as an example. The sequence analysis of the cDNA fragments revealed 65 different genes or mRNAs with higher expression levels at oestrus (see Table 1). Sixty-two genes corresponded to genes of known or inferred function, either the bovine gene or the likely human orthologue. The most pronounced differences in mRNA levels were found for *CLDN10* (claudin 10), *INHBA* (inhibin beta A), *MMP2* (matrix metalloproteinase 2), *PCSK5* (proprotein convertase 5), *RARRES1* (retinoic acid (RA) receptor responder 1), *SAL1* (salivary gland protein), *TNC* (tenascin C), *UTMP* and one unknown gene. The expression difference of these genes was termed 'on', i.e. no or very weak signals were detected at dioestrus by array hybridisation. The cDNA fragments of 25 genes were found more than once among the 444 cDNA fragments, most frequently for *SPARC* (osteonectin, 31 cDNA fragments) and *UTMP* (29 cDNA fragments).

At dioestrus, 68 genes with increased expression were detected (Table 2). Fifty-six genes corresponded to genes of known or inferred function. The genes with the highest differences in mRNA levels were *AGT* (angiotensinogen), *ATP1B2* (Na/K ATPase), *CYP26A1* (cytochrome P450 family member), *DGAT2* (diacylglycerol O-acyltransferase), *LY6 G6C* and *LY6 G6E* (lymphocyte antigen complex), *PENK* (proenkephalin), *TDGF1* (teratocarcinoma-derived growth factor 1) and a cDNA of unknown function. Again, for 25 genes more than one cDNA fragment was detected, most frequently for *PENK* (28 cDNA fragments) and *GM2A*

Figure 1 (a) Uterine sections. Endometrial tissue samples were collected from seven sections of the uterus: corpus (c) as well as caudal (ca), middle (m) and cranial (cr) parts of the ipsilateral and the contralateral uterine horns. (b) Array hybridisation. Radioactively labelled cDNA probes were prepared from the seven tissue samples of every animal (oestrus $n=3$, dioestrus $n=3$). These 42 probes were hybridised with randomly picked clones of two subtracted libraries, one for genes upregulated at oestrus and one for genes upregulated at dioestrus. Signals of one cDNA fragment of the *UTMP* gene derived from the probes of one animal at oestrus and one at dioestrus are shown.

(similar to GM2 ganglioside activator protein) (12 cDNA fragments).

In addition to the comparison of oestrus and dioestrus, the expression along the uterine horns from cranial to the corpus was investigated at the ipsilateral and the contralateral side at oestrus and dioestrus respectively. A few genes showed indeed expression differences from the cranial uterine horn to the corpus (see Table 3). Most of the genes with such expression differences showed a lower expression only in the corpus, e.g. the expression of the majority of genes was very similar across the uterine horns. The most pronounced expression gradient was observed for the *UTMP* mRNA during oestrus at both sides of the uterus. Gene expression was also analysed between the corresponding ipsilateral and contralateral sections of the uterine horns. Our recent study of the bovine oviduct (Bauersachs *et al.* 2003) and two studies of the bovine endometrium (Malayer *et al.* 1988, Williams *et al.* 1992) indicated that there could also be gene expression differences between the two uterine horns. For a few genes there was a tendency for an expression difference between the two uterine horns, but it was not significant.

For ten selected genes the expression in the endometrium was quantified by the use of real-time RT-PCR (Table 4). The same 42 RNA samples as for array hybridisation were used. All segments of the uterus

were analysed for *EPHX2* (epoxide hydrolase 2), *MGP* (matrix gla protein) and *UTMP* mRNA assuming a gradient along the uterine horns. For all other genes examined only the ipsi- and contralateral middle sections representing the total uterine horn were analysed. The mean crossing points (CP) normalised to the housekeeping gene *Ubiquitin* and the resulting expression ratios comparing oestrus and dioestrus are shown. The abundance of all investigated specific transcripts was remarkably different between the oestrus and dioestrus stage. The highest ratio between oestrus and dioestrus (150-fold) was found for the *UTMP* mRNA (cranial part of the ipsilateral uterine horn). The strong gradient of the *UTMP* mRNA abundance along the uterine horns at both sides of the uterus during oestrus was clearly confirmed, whereas for *EPHX2* and *MGP* only a tendency for a slightly higher expression in the cranial parts of the uterine horns was found. Overall the results of quantitative RT-PCR and array hybridisation correlated very well.

For five selected genes (*INHBA*, *PTHLH*, *SAL1*, *UTMP* and *PENK*) *in situ* hybridisation with bovine endometrial tissue sections were done to localise the mRNA expression in this complex tissue. A specific pattern of mRNA distribution was found for each of these genes (Fig. 2a and b). The hybridisation signal was always confined to cells of the endometrium and was

Table 1 Mean fold-change of all uterine sections for the genes upregulated at oestrus

	Gene name	GenBank	Mean	CV (%)	Ontology
Gene/cDNA/homologue description					
88128 MARC 1BOV <i>B. taurus</i> cDNA (UniGene Bt.5193, strongly similar to acid phosphatase 5, tartrate resistant (<i>H. sapiens</i>))	ACP5	AW447045	3.1 ¹	40	ECM remodelling
<i>B. taurus</i> mRNA for similar to alpha 2 actin	ACTA2	AB098797	2.4	5	Cytoskeleton
<i>B. taurus</i> β -actin	ACTB	AY141970	2.1	5	Cell motility, cytoskeleton
<i>H. sapiens</i> actin, gamma 2, smooth muscle, enteric	ACTG2	NM_001615	3.0 ²	32	Cell motility, cytoskeleton
<i>B. taurus</i> annexin A2	ANXA2	NM_174716	2.1 ²	20	ECM remodelling, cell growth
<i>B. taurus</i> apolipoprotein E	APOE	NM_173991	3.3 ¹	27	Transport, apoptosis-related function, cytoskeleton
<i>B. taurus</i> carbonic anhydrase II	CA2	NM_178572	4.6 ²	47	ECM remodelling
<i>H. sapiens</i> claudin 10	CLDN10	NM_006984	On ¹	—	Cell adhesion
<i>B. taurus</i> partial col1A1 gene for pro alpha 1(I) collagen	COL1A1	AJ312112	3.9	19	ECM structural protein
<i>B. taurus</i> collagen, type I, alpha 2	COL1A2	NM_174520	4.2 ²	5	ECM structural protein
<i>H. sapiens</i> collagen, type V, alpha 2	COL5A2	NM_000393	2.8 ¹	5	ECM structural protein
<i>M. musculus</i> procollagen, type VI, alpha 3	COL6A3	BC057903	5.1 ²	31	ECM, cell adhesion
<i>B. taurus</i> COL12 mRNA for type XII collagen	COL12A1	AB099882	5.7 ¹	23	ECM, cell adhesion
<i>H. sapiens</i> collagen, type XV, alpha 1	COL15A1	NM_001855	3.7	21	ECM structural protein
<i>H. sapiens</i> cathepsin K (pseudosclerosis)	CTSK	NM_000396	4.2 ¹	44	ECM remodelling
<i>B. taurus</i> decorin	DCN	NM_173906	1.9 ²	21	Signal transduction, ECM remodelling
<i>B. taurus</i> mRNA for similar to elongation factor-1- γ	EEF1G	AB098933	2.2 ²	15	Protein biosynthesis
<i>H. sapiens</i> fibulin 1	FBLN1	NM_001996	3.9	41	ECM, cell adhesion
<i>M. musculus</i> fibulin 5	FBLN5	NM_011812	2.6 ¹	17	ECM, cell adhesion
<i>B. taurus</i> partial mRNA for fibronectin (V+I-10)-splice variant	FN1	AJ320528	3.5 ²	19	ECM, cell adhesion
<i>B. taurus</i> gap junction protein, α 1, 43 kDa (connexin 43)	GJA1	NM_174068	3.5 ¹	9	Signal transduction, cell adhesion
<i>B. taurus</i> gastrin-releasing peptide	GRP	NM_178319	2.3	43	Signal transduction, cell growth
<i>H. sapiens</i> IGFBP-6	IGFBP6	NM_002178	2.0	8	Signal transduction, cell growth (–), proliferation (–)
<i>B. taurus</i> inhibin, beta A	INHBA	NM_174363	On ¹	—	Pregnancy, signal transduction, cell growth (–), proliferation (–), apoptosis-related function
<i>B. taurus</i> mRNA for similar to 40S ribosomal protein SA (P40) (laminin receptor 1)	LAMR1	AB099011	1.8 ²	24	Signal transduction, cell adhesion
<i>B. taurus</i> lectin, galactoside-binding, soluble, 1	LGALS1	NM_175782	2.8	22	Immune-related, signal transduction, ECM remodelling, cell growth (–), proliferation (–), apoptosis-related function, cell adhesion
<i>H. sapiens</i> melanoma antigen, family D, 1	MAGED1	NM_006986	2.4 ¹	11	Apoptosis-related function
<i>B. taurus</i> microfibril-associated glycoprotein-2 (MAGP2)	MAGP2, MFAP5	NM_174386	3.3	42	ECM structural protein
<i>H. sapiens</i> hypothetical protein MGC10540	MGC10540	NM_032353	2.5	13	—
<i>B. taurus</i> matrix metalloproteinase 2 (72 kDa type IV collagenase)	MMP2	NM_174745	On ²		ECM remodelling, pregnancy, hydrolase
<i>H. sapiens</i> mucin 16	MUC16	AF361486	3.8 ²	41	Immune-related
<i>H. sapiens</i> Norrie disease (pseudoglioma)	NDP	NM_000266	4.8	11	Signal transduction, cell growth, Proliferation
<i>B. taurus</i> nephroblastoma overexpressed protein precursor gene	NOV	AF055076	7.0	27	Signal transduction, cell motility, cell adhesion, cell growth

Continued

Table 1 continued

	Gene name	GenBank	Mean	CV (%)	Ontology
<i>H. sapiens</i> nucleophosmin (nucleolar phosphoprotein B23, numatrin)	NPM1	NM_002520	1.7 ²	22	Proliferation, apoptosis-related function
<i>B. taurus</i> procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide	P4HB	NM_174135	2.7	10	ECM remodelling
<i>H. sapiens</i> procollagen C-endopeptidase enhancer	PCOLCE	NM_002593	2.6	18	ECM remodelling
<i>H. sapiens</i> proprotein convertase subtilisin/kexin type 5	PCSK5	NM_006200	On ¹	—	ECM remodelling, hydrolase
<i>H. sapiens</i> pyruvate kinase, muscle	PKM2	NM_002654	1.9	18	Glycolysis
<i>B. taurus</i> peroxiredoxin 2	PRDX2	NM_174763	2.1 ¹	9	Oxidoreductase
<i>H. sapiens</i> protease, serine, 11 (IGF binding)	PRSS11	NM_002775	2.6 ²	34	Signal transduction, cell growth (–), hydrolase
<i>H. sapiens</i> patched homologue 2 (<i>Drosophila</i>)	PTCH2	AY438664	2.5 ²	43	Signal transduction, proliferation (–)
<i>B. taurus</i> parathyroid hormone-like hormone	PTH1H	NM_174753	6.3 ¹	51	Signal transduction, proliferation, pregnancy
<i>H. sapiens</i> transcription factor RAM2	RAM2	NM_018719	2.7	21	Transcriptional regulation
<i>H. sapiens</i> RA receptor responder (tazarotene induced) 1	RARRES1	NM_206963	On	—	Proliferation (–), cell adhesion
<i>H. sapiens</i> regenerating islet-derived family, member 4	REG4	NM_032044	2.2	27	Proliferation
<i>B. taurus</i> mRNA for similar to ribosomal protein L18a	RPL18A	AB098916	2.1 ²	15	Protein biosynthesis
<i>H. sapiens</i> ribosomal protein L7a	RPL7A	NM_000972	2.1 ²	19	Protein biosynthesis
<i>B. taurus</i> mRNA for similar to S100 calcium-binding protein A11	S100A11	AB099012	2.7	6	Proliferation (–), cell motility
CES014220 <i>B. taurus</i> skin cDNA library <i>B. taurus</i> cDNA (UniGene Bt.13796)	SAL1	CF769307	On	—	Signal transduction, transport, pregnancy
4059330 BARC 8BOV <i>B. taurus</i> cDNA (UniGene Bt.21836)	SEC61A1	CK834803	2.2 ²	8	Transport
<i>B. taurus</i> serine (or cysteine) proteinase inhibitor, clade A, member 1	SERPINA1	NM_173882	3.0	14	Immune-related
<i>B. taurus</i> serine (or cysteine) proteinase inhibitor, clade F, member 1	SERPINF1	NM_174140	2.4	11	Proliferation, apoptosis-related function
<i>H. sapiens</i> serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	NM_001235	4.3 ²	26	ECM remodelling
<i>H. sapiens</i> SRY (sex determining region Y)-box 4	SOX4	NM_003107	2.6 ¹	12	Transcriptional regulation
<i>B. taurus</i> secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	NM_174464	3.2	3	ECM structural protein, ECM remodelling, cell motility, proliferation (–)
<i>H. sapiens</i> Tax interaction protein 1	TAX1BP3	NM_014604	2.3 ¹	13	Signal transduction, proliferation (–)
<i>B. taurus</i> thrombospondin	THBS1	NM_174196	1.7 ¹	35	ECM, cell adhesion, cell motility, signal transduction
<i>H. sapiens</i> tenascin C (hexabrachion)	TNC	NM_002160	On ¹	—	Cell adhesion, ECM structural protein, immune-related
<i>H. sapiens</i> tubulin, alpha 1 (testis specific)	TUBA1	NM_006000	2.1	26	Cytoskeleton
<i>H. sapiens</i> beta 5-tubulin (OK/SW-cl.56)	TUBB	NM_178014	2.4 ¹	26	Cytoskeleton
<i>B. taurus</i> uterine milk protein precursor	UTMP, LOC286871	NM_174797	On ³	—	Immune-related, pregnancy
<i>B. taurus</i> vimentin	VIM	NM_173969	2.0 ²	29	Cytoskeleton, immune-related
<i>H. sapiens</i> zinc finger protein 564	ZNF564	BC036481	2.5 ²	40	Transcriptional regulation
4062746 BARC 8BOV <i>B. taurus</i> (UniGene Bt.13993)	—	CK837495	3.0 ¹	6	—
Genomic scaffold 246248	—	AAFC01272447	On	—	—

Gene name: official gene name/symbol from Entrez Gene; On: no expression detected at dioestrus; ¹ no expression detected in the corpus of one pair at dioestrus and oestrus; ² inconsistent expression in the corpus; ³ no expression detected in the corpus of all animals; (–) negative regulation; (+) positive regulation

Table 2 Mean fold-change of all uterine sections for the genes upregulated at dioestrus

	Gene name	GenBank	Mean	CV (%)	Ontology
Gene/cDNA/homologue description					
<i>H. sapiens</i> ankyrin repeat and BTB (POZ) domain containing 1	ABTB1	NM_172027	2.2	26	Cell growth (–), proliferation (–)
<i>Ovis ammon</i> mRNA for angiotensinogen	AGT	D17520	On	—	Pregnancy
20 alpha-hydroxysteroid dehydrogenase (cattle)	AKR1B1	S54973	6.0	15	Dehydrogenase
Alkaline phosphatase tissue non-specific isoform/TNS-AP (cattle)	ALPL	S81600	4.8	10	ECM remodelling, hydrolase
<i>B. taurus</i> Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	NM_175797	5.9	9	Signal transduction, immune-related, cell adhesion (–)
Human adult mRNA for Na/K ATPase beta 2 subunit	ATP1B2	D87330	On	—	Transport (ion channel)
503071 MARC 2BOV <i>B. taurus</i> cDNA (UniGene Bt.24822, moderately similar to NP_059345-BAI1-associated protein 2, isoform 2)	BAIAP2	BM089351	3.1 ¹	21	Receptor activity
721820 MARC 6BOV <i>B. taurus</i> cDNA (UniGene Bt.17278, strongly similar to NP_005495 branched chain aminotransferase 1, cytosolic)	BCAT1	CB461808	5.3	11	Proliferation (+), transferase
<i>H. sapiens</i> chromosome 5 open reading frame 18	C5orf18	NM_005669	3.6	3	—
<i>H. sapiens</i> cyclin B1	CCNB1	NM_031966	3.3	13	Cell cycle related (+)
<i>H. sapiens</i> chloride channel Ka	CLCNKA	NM_004070	5.6	35	Transport (ion channel)
<i>H. sapiens</i> CCR4-NOT transcription complex, subunit 1	CNOT1	NM_016284	3.0	10	—
<i>H. sapiens</i> carboxypeptidase N, polypeptide 1, 50 kDa	CPN1	NM_001308	4.7	16	Proteolysis, hydrolase
<i>H. sapiens</i> cytochrome P450, family 26, subfamily A, polypeptide 1	CYP26A1	NM_000783	On	—	Pregnancy, transport
<i>B. taurus</i> DGAT2 gene for diacylglycerol O-acyltransferase 2	DGAT2	AJ534372	On	—	Transferase
<i>H. sapiens</i> delta sleep inducing peptide, immunoreactor	DSIPI	NM_198057	5.1	12	Immune-related, transcriptional regulation
<i>H. sapiens</i> embryonic ectoderm development	EED	NM_152991	2.8	31	Signal transduction, transcriptional regulation, pregnancy
<i>H. sapiens</i> ephrin-A1	EFNA1	NM_004428	3.2	43	Signal transduction, kinase
<i>H. sapiens</i> epoxide hydrolase 2, cytoplasmic	EPHX2	NM_001979	7.5	32	Immune-related, hydrolase
<i>H. sapiens</i> RNA-binding protein	FLJ20273	NM_019027	2.9	12	—
603116 MARC 6BOV <i>B. taurus</i> cDNA (UniGene Bt.17182, moderately similar to NP_005259-1 gap junction protein, beta 5)	GJB5	CB427531	3.1	37	Transport (channel)
Similar to <i>H. sapiens</i> GM2 ganglioside activator protein	GM2A	NM_000405	9.8	43	Glycolipid catabolism
<i>H. sapiens</i> helicase with zinc finger domain	HELZ	NM_014877	3.7	31	Helicase activity, nucleic acid binding
<i>Bubalus</i> sp. mRNA for NAD ⁺ -dependent 15-hydroxyprostaglandin dehydrogenase	HPGD	AJ222837	6.7	40	Pregnancy, oxidoreductase, dehydrogenase
<i>B. taurus</i> isocitrate dehydrogenase 1 (NADP ⁺), soluble	IDH1	NM_181012	4.0	36	Oxidoreductase, dehydrogenase
<i>B. taurus</i> mRNA for immunoglobulin light chain	IG G1	X62917	8.1	58	Immune-related (antibody)
<i>H. sapiens</i> integrin, beta 4	ITGB4	NM_000213	2.8	16	Cell adhesion, ECM remodelling, signal transduction, kinase
<i>H. sapiens</i> hypothetical protein BC001573	LOC134147	NM_138809	4.2	41	Hydrolase
971446 BARC 5BOV <i>B. taurus</i> cDNA (UniGene Bt.8371, moderately similar to low-density lipoprotein receptor-related protein 2)	LRP2	CK848654	8.0	18	Transport
148109 MARC 4BOV <i>B. taurus</i> cDNA (UniGene Bt.2346, moderately similar to NP_002337 lymphocyte antigen 6 complex, locus E)	LY6E	BE664006	3.6	18	Immune-related
<i>H. sapiens</i> lymphocyte antigen 6 complex, locus G6C	LY6G6C	NM_025261	On	—	Immune-related

Continued

Table 2 Continued

	Gene name	GenBank	Mean	CV (%)	Ontology
4080472 BARC 9BOV <i>B. taurus</i> cDNA clone (UniGene Bt.9683, similar to lymphocyte antigen 6 complex, locus G6E)	LY6G6E	CK965552	On ²	—	Immune-related, signal transduction
<i>H. sapiens</i> cDNA FLJ37829 fis, weakly similar to <i>H. sapiens</i> mRNA for mucolipidin (UniGene Hs.535239 mucolipin 3)	MCOLN3	AK095148	4.8	48	Transport
<i>H. sapiens</i> hypothetical protein MGC15429	MGC15429	NM_032750	2.4	9	Catalytic activity
<i>H. sapiens</i> clone DNA176108 scavenger receptor hlg (UNQ2938)	MGC45780	AY358150	21.1	9	Receptor activity
<i>B. taurus</i> matrix Gla protein	MGP	NM_174707	4.5	19	ECM structural protein, ECM remodelling
<i>H. sapiens</i> membrane-spanning 4-domains, subfamily A, member 8B	MS4A8B	NM_031457	5.3	52	Signal transduction
<i>H. sapiens</i> myotubularin related protein 3	MTMR3	NM_153050	6.4	23	Signal transduction
<i>M. musculus</i> N-myc downstream regulated 4	NDRG4	NM_145602	4.9	38	Cell growth (+), hydrolase
968490 MARC 4BOV <i>B. taurus</i> cDNA 3' (UniGene Hs.5025, <i>H. sapiens</i> nebullette)	NEBL	CK846036	6.7	13	Actin-binding
4113753 BARC 9BOV <i>B. taurus</i> cDNA (UniGene Bt.14398, moderately similar to phosphatidylethanolamine-binding protein)	PBP	CK940308	3.6	26	Signal transduction
<i>H. sapiens</i> phosphatidylethanolamine-binding protein 4	PEBP4	NM_144962	3.1	32	—
<i>B. taurus</i> proenkephalin	PENK	NM_174141	On	—	Signal transduction, pregnancy
<i>H. sapiens</i> phospholipase A2, group X	PLA2G10	NM_003561	5.2	12	Lipid catabolism, hydrolase
725142 MARC 6BOV <i>B. taurus</i> cDNA (UniGene Bt.1286, moderately similar to NP_002880 retinoic acid receptor responder 2)	RARRES2	CB463961	2.8	26	Cell growth, proliferation
<i>H. sapiens</i> retinal short chain dehydrogenase reductase	RDHE2	NM_138969	4.9	40	Dehydrogenase
4107201 BARC 9BOV <i>B. taurus</i> cDNA clone 9BOV33_L08 5' (UniGene Hs.445030 Rho-related BTB domain containing 3)	RHOBTB3	CK976167	2.5	30	Signal transduction
<i>B. taurus</i> RTN3 (reticulon 3)	RTN3	BK001797	3.9	34	—
<i>H. sapiens</i> secernin 1	SCRN1	NM_014766	2.9	53	Immune-related
<i>B. taurus</i> selenoprotein P-like protein precursor	SEPP1	NM_174459	2.9	32	Transport
<i>H. sapiens</i> serum/glucocorticoid regulated kinase	SGK	NM_005627	2.1	20	Signal transduction, kinase, transferase, apoptosis, transport
<i>H. sapiens</i> solute carrier family 16 (monocarboxylic acid transporters), member 11	SLC16A11	NM_153357	7.6	21	Transport
<i>H. sapiens</i> SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily b, member 1	SMARCB1	NM_003073	7.8	48	Cell cycle related, transcriptional regulation
<i>H. sapiens</i> SRY (sex determining region Y)-box 17	SOX17	NM_022454	3.0	25	Transcription regulation, pregnancy
<i>H. sapiens</i> teratocarcinoma-derived growth factor 1	TDGF1	NM_003212	On		Signal transduction, cell growth (+)
<i>B. taurus</i> transglutaminase 2	TGM2	NM_177507	4.4	10	Apoptosis related, transferase
<i>B. taurus</i> tissue inhibitor of metalloproteinase 2	TIMP2	NM_174472	5.5	12	ECM remodelling, pregnancy
<i>H. sapiens</i> transducin-like enhancer of split 1 (E(sp1) homologue, <i>Drosophila</i>)	TLE1	NM_005077	3.2	13	Signal transduction, transcriptional regulation
4070793 BARC 10BOV <i>B. taurus</i> cDNA (UniGene Bt.28503)	—	CK946314	4.9	28	—
4081167 BARC 9BOV <i>B. taurus</i> cDNA (UniGene Bt.36133)	—	CK965794	2.7	16	—
4082241 BARC 9BOV <i>B. taurus</i> cDNA (UniGene Bt.29337)	—	CK966960	4.8	34	—

Continued

Table 2 Continued

	Gene name	GenBank	Mean	CV (%)	Ontology
4082241 BARC 9BOV <i>B. taurus</i> cDNA (UniGene Bt.29337)	—	CK966960	4.8	34	—
604955 MARC 6BOV <i>B. taurus</i> cDNA (UniGene Bt.24986)	—	CB429159	2.3	30	—
776530 MARC 6BOV <i>B. taurus</i> cDNA (UniGene Bt.12715)	—	CB538542	On	—	—
<i>H. sapiens</i> mRNA; cDNA DKFZp667B1913 (UniGene Hs.374451)	—	AL832403	3.0	28	—
<i>H. sapiens</i> mRNA; cDNA DKFZp761L1121 (UniGene Hs.171939)	—	AL713639	6.8	29	—
<i>H. sapiens</i> mRNA; cDNA DKFZp761M0111 (UniGene Hs.368337)	—	AL137346	2.4	22	—
Genomic Scaffold 110056	—	AAFC01187078	6.1	37	—
Genomic Scaffold 289710	—	AAFC01019020	3.8	75	—

Gene name: official gene name/symbol from Entrez Gene; On: no expression detected at oestrus; 1 inconsistent expression in the corpus; 2 no expression detected in all uterine sections of one animal pair at dioestrus and oestrus; (–) negative regulation; (+) positive regulation

absent in the myometrium and the serosa. No specific signal was observed in sections hybridised with the sense strand (Fig. 2c) or in sections incubated with buffer only

instead of the oligonucleotide probes. Considerable levels of the *INHBA* message were detected in the supranuclear area of the surface epithelium. The

Table 3 Gradients of gene expression from the cranial uterine horn to the corpus

	Gene name	GenBank	Gradient	cran	mid	caud	corp	Pattern
Gene/cDNA description								
88128 MARC 1BOV <i>B. taurus</i> cDNA (UniGene Bt.5193, strongly similar to acid phosphatase 5, tartrate resistant (<i>H. sapiens</i>))	ACP5	AW447045	Es ip	4.3	1.9	1.5	1.0	2111
			Es co	4.1	2.7	1.5	1.0	3211
<i>Ovis ammon</i> mRNA for angiotensinogen	AGT	D17520	Di co	3.1	1.7	1.8	1.0	2111
<i>H. sapiens</i> epoxide hydrolase 2, cytoplasmic	EPHX2	NM_001979	Di ip	2.3	1.8	2.0	1.0	2221
		qPCR	Di ip	1.6	1.1	1.4	1.0	—
			Di co	2.7	2.0	1.6	1.0	3221
		qPCR	Di co	1.6	1.2	1.3	1.0	—
Similar to <i>H. sapiens</i> GM2 ganglioside activator protein	GM2A	NM_000405	Di ip	3.2	2.5	2.8	1.0	2221
			Di co	3.2	2.7	2.2	1.0	2221
<i>B. taurus</i> IGFBP-4	IGFBP4	NM_174557	Es ip	3.9	2.2	2.2	1.0	3221
			Es co	2.8	1.9	1.9	1.0	2221
<i>H. sapiens</i> integrin, beta 4	ITGB4	NM_000213	Di co	2.5	1.8	1.6	1.0	3221
<i>M. musculus</i> N-myc downstream regulated 4	NDRG4	NM_145602	Di ip	2.8	2.6	2.7	1.0	2221
			Di co	3.9	3.4	2.8	1.0	2221
<i>H. sapiens</i> phosphorylase kinase, gamma 2	PHKG2	NM_000294	Di ip	3.2	3.0	3.4	1.0	2221
<i>B. taurus</i> uterine milk protein precursor	UTMP	NM_174797	Es ip	30.6	6.7	3.2	1.0	2111
		qPCR	Es ip	34.5	9.1	2.3	1.0	—
			Es co	20.5	6.6	3.7	1.0	4321
		qPCR	Es co	20.5	11.4	5.3	1.0	—
4100874 BARC 10BOV <i>B. taurus</i> cDNA clone 10BOV7_D17 (UniGene Bt.20233)	—	CK960198	Di ip	3.1	2.8	3.0	1.0	2221
<i>B. indicus</i> BoLA DQB gene for first domain of MHC class 2 molecule, beta chain	—	Z48207	Es ip	2.7	2.2	2.2	1.0	2221
			Es co	2.5	2.5	2.4	1.0	2221
			Di co	3.1	1.9	1.3	1.0	2111

Gradient: Es ip: oestrus, ipsilateral; Es co: oestrus contralateral; Di ip: dioestrus, ipsilateral; Di co: dioestrus contralateral; Pattern: pattern represents significance of signal differences revealed by the post-hoc test

Table 4 Results of quantitative real-time RT-PCR

Mean crossing points (n=3) normalised to ubiquitin

	Oestrus							Dioestrus						
	i-cr	i-m	i-ca	c-cr	c-m	c-ca	C	i-cr	i-m	i-ca	c-cr	c-m	c-ca	C
Upregulated at oestrus														
CLDN	—	—	—	—	—	—	—	—	—	—	—	—	—	—
INHBA	—	20.9	—	—	20.8	—	—	—	26.2	—	—	26.0	—	—
PTHLH	—	22.9	—	—	22.7	—	—	—	28.1	—	—	27.7	—	—
SOX4	—	23.3	—	—	23.3	—	—	—	27.2	—	—	27.0	—	—
UTMP	—	22.6	—	—	22.7	—	—	—	24.6	—	—	24.6	—	—
19.6	19.6	21.5	23.5	20.3	21.2	22.3	24.7	26.8	27.4	27.4	26.6	27.1	27.4	27.4
Upregulated at dioestrus														
CPN1	—	—	—	—	—	—	—	—	—	—	—	—	—	—
EPHX2	—	28.6	—	—	28.7	—	—	—	26.8	—	—	26.7	—	—
MGP	26.6	26.6	26.9	26.6	26.6	26.7	26.7	22.8	23.2	22.9	22.7	23.1	23.0	23.4
MS4A8B	26.8	26.8	26.7	26.7	26.8	27.0	27.0	24.4	24.4	24.3	24.0	24.2	24.1	24.7
PENK	—	23.3	—	—	23.3	—	—	—	21.3	—	—	21.5	—	—
24.9	24.9	—	—	—	25.2	—	—	—	20.0	—	—	19.9	—	—
Housekeeping gene														
Ubiquitin	18.3	18.3	18.3	18.0	18.2	18.3	18.7	18.0	17.8	17.9	18.0	18.0	18.1	17.9

Expression ratios between oestrus and dioestrus

	i-cr			i-m			i-ca			c-cr			c-m			c-ca			C		
	FC	P		FC	P		FC	P		FC	P		FC	P		FC	P		FC	P	
Upregulated at oestrus																					
CLDN	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
INHBA	—	—	—	39.2	<0.01	—	—	—	—	—	—	—	37.2	<0.01	—	—	—	—	—	—	—
PTH1H	—	—	—	36.8	<0.01	—	—	—	—	—	—	—	32.4	<0.01	—	—	—	—	—	—	—
SOX4	—	—	—	15.0	<0.01	—	—	—	—	—	—	—	13.6	<0.01	—	—	—	—	—	—	—
UTMP	—	—	—	4.2	<0.01	—	—	—	—	—	—	—	3.6	<0.01	—	—	—	—	—	—	—
149.4	149.4	<0.01	61.2	<0.01	15.4	0.04	81.0	<0.01	<0.01	<0.01	<0.01	<0.01	62.4	<0.01	<0.01	35.9	<0.01	<0.01	6.8	0.17	0.17
Upregulated at dioestrus																					
CPN1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
EPHX2	—	—	—	3.5	<0.01	—	—	—	—	—	—	—	4.0	<0.01	—	—	—	—	—	—	—
MGP	14.2	<0.01	10.6	<0.01	<0.01	15.6	<0.01	<0.01	<0.01	14.6	<0.01	<0.01	11.6	<0.01	<0.01	12.9	<0.01	<0.01	10.3	<0.01	<0.01
MS4A8B	5.3	0.06	5.3	0.03	0.03	5.4	0.03	0.03	0.04	6.4	0.04	0.04	6.1	0.02	0.02	7.4	0.02	0.02	5.0	0.03	0.03
PENK	—	—	—	3.9	<0.01	—	—	—	—	—	—	—	3.6	<0.01	—	—	—	—	—	—	—
30.2	30.2	<0.01	30.2	<0.01	<0.01	—	—	—	—	—	—	—	38.0	<0.01	<0.01	—	—	—	—	—	—
Housekeeping gene																					
Ubiquitin	1.2	—	1.5	—	—	1.4	—	—	—	1.7	—	—	1.2	—	—	1.2	—	—	1.0	—	—

i-cr, ipsilateral cranial; i-m, ipsilateral middle; i-ca, ipsilateral caudal; c-cr, contralateral cranial; c-m, contralateral middle; c-ca, contralateral caudal; C, corpus; FC: fold-change; P, P-value

Figure 2 *In situ* hybridisation. Tissue samples of the middle part of the ipsilateral uterine horn were used from animals slaughtered at oestrus for the detection of *INHBA*, *PTHLH*, *SAL1* and *UTMP* mRNAs or at dioestrus for *PENK* mRNA. Endometrial sections near the epithelial surface (a), of the deep uterine glands (b), and hybridised with the corresponding sense oligonucleotide (c) are shown.

superficial uterine glands also showed a distinct staining of several cells within a cross-section of the gland. In the

deep uterine glands adjacent to the myometrium, the *INHBA* message appeared somewhat reduced, but was

still more pronounced in the apical area of the glandular epithelium. Most of the fibroblasts in the endometrium displayed a weak hybridisation signal. High levels of *PTHLH* mRNA were found in the uterine surface epithelium and in the deep uterine glands. Also the fibroblasts of the endometrium showed a distinct staining. In the superficial uterine glands, the hybridisation signal was less pronounced and appeared concentrated to the supranuclear area of the glands. Strong staining for *SALI* mRNA could be demonstrated in the uterine surface epithelium and in the supranuclear epithelial area of the superficial uterine glands. Deep uterine glands stained generally more weakly, with a distinct signal in the apical region of the glands. mRNA for *UTMP* was detected in dispersed epithelial cells of the surface epithelium but distinct staining was found in the epithelium of the superficial and deep uterine glands. *PENK* was weakly expressed in the surface epithelium but the signal intensity increased in the uterine glands.

To deduce a picture of the regulatory processes that proceed during the oestrous cycle in the endometrium from the changes at the mRNA level, two bioinformatic approaches were applied. At first, a functional classification of the identified genes was done by building a simplified Gene Ontology (GO) for genes upregulated either at oestrus (see Table 1, Ontology) or at dioestrus (see Table 2, Ontology). For a graphical illustration the numbers of genes assigned to the 19 functional groups at oestrus and dioestrus are compared in Fig. 3. Some biological processes or molecular functions clearly dominated either at oestrus or at dioestrus. At oestrus the functional groups 'Cytoskeleton', 'Cell motility', 'Cell adhesion', 'Extracellular matrix (ECM) structural proteins', 'ECM remodelling' and 'Proliferation' clearly prevail. At dioestrus, genes of several groups of enzymes and 'Transport' are more upregulated.

Furthermore, involved pathways and signalling cascades were identified by the analysis of KEGG pathways and a literature search. Thirteen pathways and the assigned genes at oestrus and dioestrus are listed in Table 5. The highest number of genes (17 genes) was assigned to the transforming growth factor- β (TGF- β) signalling pathway. Most of these genes (14 genes) were upregulated at oestrus and corresponded to target genes or to inhibitors of this pathway. In Fig. 4 a simplified TGF- β signalling pathway based on the *Homo sapiens* reference pathway derived from the KEGG pathway database with the assigned genes is shown. For the Prostaglandin metabolism (four genes) as well as for the Phosphoinositide signalling (four genes) only such genes could be assigned that were upregulated at dioestrus. In contrast, for the Phospholipase C pathway (three genes) and also for the Ribosome pathway (four genes) only such genes were found that were upregulated at oestrus.

Finally, a comparison of the genes found in this and other studies of the human, the non-human primate

Figure 3 Comparison of the simplified GO at oestrus and dioestrus. For the differentially expressed genes of known or inferred function a simplified GO was built. The number of genes is shown for every GO group at oestrus (black bars) and at dioestrus (grey bars) respectively.

(Rhesus monkey), and the mouse system (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.* 2003, Riesewijk *et al.* 2003, Tan *et al.* 2003, Ace & Okulicz 2004) was done (Table 6). The cycle stages that were compared in these studies are stated in the footnotes of Table 6. The overall overlap with these studies was 34 genes. Considering closely related genes of the same gene family the overlap was even higher (data not shown).

Table 5 Assignment to cellular pathways

Pathway	Upregulated at oestrus	Function	Upregulated at dioestrus	Function
TGF-beta signalling pathway (hsa04350) ¹	DCN	Inhibition (hsa04350)	LRP2	Endocytosis, downregulated by TGFβ1
	INHBA	Inhibition (hsa04350)	MGP	Binding protein of BMP2
	THBS1	Inhibition (hsa04350)	TDGF1	Blocks INHBA signalling; ligand and coreceptor in nodal signalling pathway
	COL1A1/2, 5A2, 6A3	Target genes		
	FBLN5	Target gene		
	FN1	Regulated by TGF-β in endometrial cells		
	PRSS11, S100A11, SPARC	Inhibition of TGFβ1 and BMP Growth inhibition Coupled to TGFβR- and Smad2/3-dependent pathway		
MAPK signalling pathway (hsa04010)	TNC	Target gene		
	UTMP	Binding of INHBA		
	GJA1	Inhibited by LH via both PKA and MAPK cascades	AKR1B1	Upregulated by oxidative stress via EGFR-ERK pathway/p38 kinase pathway
	LAMR1	MAPK are involved in the laminin receptor signal transduction pathway	DSIPI	Inhibits Raf-1 phosphorylation resulting in suppression of MEK/ERK-1/2 phosphorylation and AP1-dependent transcription
	PTH1H	PI-3-kinase pathway may be involved in PTHrP stimulation of Ras, MAPK pathway, upregulation of COL1	EFNA1	Activation of Eck (EPHA2)
	DCN	Suppressed by activation of the ERK1,2 signalling pathway in fibroblasts	PBP	Inhibitor of the Raf/MAP kinase signalling cascade (Raf-1/MEK/ERK pathway)
			PLA2G10	MAPK signalling pathway (hsa04010)
RA signalling/metabolism	LGALS1	RA-induced differentiation	CYP26A1	Inactivates RA
	RARRES1, SERPINH1	Induced by RA Induced by RA	RARRES2, RDHE2, TGM2	Induced by RA RA metabolism RA signalling
cAMP signalling pathway	COL1A1; COL1A2	Inhibited by parathyroid hormone via the cAMP signalling pathway	RARRES2	Ligand of GPCR CHEMR23 (CMKLR1)
	INHBA	Component of the cAMP pathway leading to endometrial stromal decidualisation	SGK	Regulated by multiple protein kinases, including PKA
Wnt signalling pathway (hsa04310)	PTH1H	cAMP signalling pathway		
	NDP	Activation of the classical Wnt pathway	SOX17	Interaction with beta-catenin
	TAX1BP3	Negative regulation of cell proliferation	TLE1	Wnt/Wg signalling
Immunoregulation	VIM	Target of the beta-catenin/TCF pathway		
	LGALS1	Regulator of inflammatory response	CPN1	Inactivates C3a and C4a and reduces C5a
	UTMP	Immunosuppression	DSIPI	Glucocorticoid hormone-mediated immunosuppression
IGF system	SERPINA1	Complement and coagulation cascades (hsa04610)		
	IGFBP6	IGF-binding protein	BAIAP2	Insulin/IGF-I signalling
	PCSK5, PRSS11	Cleavage of IGF-I Cleavage of IGFBP-5		

Continued

Table 5 Continued

	Upregulated at oestrus	Function	Upregulated at dioestrus	Function
Prostaglandin and leukotriene metabolism (hsa00590)		PLA2G10 EPHX2	AKR1B1	Glycerolipid metabolism (hsa00561)
			hsa00590 Prostaglandin metabolism HPGD	Prostaglandin metabolism
Ribosome (hsa03010)	RPL7A RPL18A LAMR1 NPM1 TNC	hsa03010 hsa03010 hsa03010 Ribosome biogenesis pathway Negative regulation of Rho-ROCK pathway via EGFR pathway	TIMP2	Suppresses EGF-mediated mitogenic signalling
EGFR pathway			AKR1B1	Upregulated by oxidative stress via EGFR-ERK/p38 kinase pathway
Sonic hedgehog signalling	PTCH2	Sonic hedgehog/PTCH signalling pathway	LRP2	Regulatory component
Phospholipase C pathway	PTHLH GRP	Hedgehog signalling pathway Stimulation of the GRP receptor activates phospholipase C pathway		
	MMP2	Generation is mediated by activation of phospholipase A(2)		
	PTHLH	Gq-mediated phospholipase C/Ca ²⁺ signalling, apoptosis		
Phosphoinositide signalling pathway			SGK	Component of PI-3-kinase signalling pathway
			EFNA1 MTMR3	Increased PI-3-kinase activity Phosphoinositide signalling

¹ ID of the *H. sapiens* reference pathway

Thirteen genes showed a similar regulation, the regulation of 17 genes was contrary, and four genes were mentioned as 'expressed' in the study of Borthwick *et al.* (2003).

Discussion

The aim of this study was the identification of differentially expressed genes in the bovine endometrium during the oestrous cycle, in particular between the late oestrus and the dioestrus stage to uncover regulatory pathways involved in the control of the complex cycle-dependent changes in this tissue. Therefore, a combination of SSH and cDNA microarrays was used. This resulted in a relatively large number of genes differentially expressed between oestrus and dioestrus (133 genes) and also a couple of genes with clear expression differences between the uterine sections. The number of differentially expressed genes between the oestrus and the dioestrus stage was comparable with that found in similar studies in the mouse, the Rhesus monkey and in humans, which have been done with

high-density cDNA or oligonucleotide arrays respectively (Borthwick *et al.* 2003, Tan *et al.* 2003, Ace & Okulicz 2004).

Using quantitative real-time RT-PCR the results of the array hybridisation were clearly confirmed. The correlation of the data was very good, although microarray data usually do not provide exact quantitative expression differences as quantitative PCR. Reasons for minor deviations in expression ratios have already been discussed elsewhere (Bauersachs *et al.* 2004). The analysis of genes with high expression ratios between oestrus and dioestrus could be done very precisely by real-time RT-PCR, extending the qualitative array result 'on' referring to no signal at one cycle phase because of too low expression signals. For instance, the expression of *UTMP* mRNA in the ipsilateral cranial endometrium at oestrus was almost 150-fold higher than at dioestrus.

The classification of the identified genes based on GOs revealed that upregulation of mRNAs of distinct functional groups clearly dominated at oestrus or dioestrus. At oestrus the striking upregulation of mRNAs for proteins of the ECM and for proteins involved in

Figure 4 Genes involved in the TGF- β signalling pathway. Differentially expressed genes of known or inferred function were assigned to the TGF- β signalling pathway (based on the *H. sapiens* reference pathway hsa04350) by direct searching this pathway and by a literature search. Grey boxes: genes of the original pathway; white boxes/solid line: genes directly assigned to the pathway; white boxes/dashed line: genes assigned by literature search; bold style: upregulated at oestrus; italics: upregulated at dioestrus; for gene symbols in grey boxes see Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi).

ECM remodelling indicates changes in the composition of the connective tissue during the oestrous cycle as proposed by Curry & Osteen (2001). Furthermore, upregulation of mRNAs coding for proteins that negatively regulate cell growth and proliferation, and with apoptosis-related functions prevailed at oestrus. For proliferation-related/inhibiting genes these changes may reflect the end of the 'proliferative phase' of the endometrium since the animals representing day 0 were slaughtered at late oestrus.

At dioestrus, a number of transcripts for different enzymes and for proteins involved in transport processes are upregulated. The cyclic regulation of uterine fluids in the endometrium by differential expression of genes coding for ion channels was first shown by Chan *et al.*

(2002). The identification of three mRNAs coding for ion channels (group 'transport') provides further evidence for this. The upregulation of many mRNAs for enzymes may be to some extent an indication for the increase of the prostaglandin metabolism at dioestrus (Goff 2004, Spencer *et al.* 2004).

The genes of known function were further analysed in the context of different pathways where they are involved. The most prominent pathway was the TGF- β signalling with 17 genes upregulated either at oestrus or at dioestrus. At oestrus, transcripts of target genes of this signalling pathway and transcripts for proteins that are shown to inhibit TGF- β signalling were upregulated. In contrast, at dioestrus only three genes were identified which are described as being involved in TGF- β

Table 6 Comparison with similar studies in humans, Rhesus monkeys and mice

Gene description	Gene name	Gene ID	Oe:Di ¹	Ace & Okulicz ²	Borthwick et al. ³	Carson et al. ⁴	Kao et al. ⁵	Riesewijk et al. ⁶	Tan et al. ⁷
Actin, alpha 2	ACTA2	59	—	—	—	—	—	—	—
Actin, beta	ACTB	60	2.4	—	—	—	—	—4	—
Annexin A2	ANXA2	302	2.1	—	e	—	—4.7	—4	—
Apolipoprotein E	APOE	348	3.3	—	e	—	—100	—	—
Collagen, type I, alpha 2	COL1A2	1278	4.2	—	e	—	—	—	—2.4
Collagen, type V, alpha 2	COL5A2	1290	2.8	2.0	—	—	—	—	—
Collagen, type VI, alpha 3	COL6A3	1293	5.1	8.0	—	—	—	—	—
Eukaryotic translation elongation factor 1 gamma	EEF1G	1937	2.2	—	e	—	—	—	—
Fibulin 5	FBLN5	10516	2.6	—	—	—	—	—6	—
Laminin receptor 1 (ribosomal protein SA, 67 kDa)	LAMR1	3921	1.8	—	—	—	—	—	—2.2
Lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	3956	2.8	2.6	e	—	—	—	—
Nephroblastoma overexpressed gene	NOV	4856	7.0	—	—5.5	—	—	—	—
Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta	P4HB	5034	2.7	—	e	—	—	—	—
Procollagen C-endopeptidase enhancer	PCOLCE	5118	2.6	—	—	—2.7	—	—	—
Proprotein convertase subtilisin/kexin type 5	PCSK5	5125	Oe on	—	6.7	—	—	—	—
RA receptor responder 1	RARRES1	5918	Oe on	—	—	—	—5	—	—
Serine (or cysteine) proteinase inhibitor, clade A, member 1	SERPINA1	5265	3.0	—	5.1	—	—	—	7.0
SRY (sex determining region Y)-box 4	SOX4	6659	2.6	3.7	—	—	—	—	—
Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	6678	3.2	3.2	e	—	—	—	—
Tenascin C (hexabrachion)	TNC	3371	Oe on	—	5.9	—	3.9	—	—
Tubulin, alpha 1	TUBA1	7277	2.1	—	—	—	—3.4	—16	—2.6
Tubulin, beta polypeptide	TUBB	7280	2.4	—	—	—	—	—	—2.0
Vimentin	VIM	7431	2.0	—	e	—	—	—	—
Alkaline phosphatase	ALPL	249	—4.8	—	—	—	—	11	—
BAI1-associated protein 2	BAIAP2	10458	—3.1	—	—	—	3.2	—	—
Cyclin B1	CCNB1	891	—3.3	—	—	—	2.5	3	—
Cytochrome P450, family 26, subfamily A, polypeptide 1	CYP26A1	1592	Di on	—	—15.6	—	—	—	—
Ephrin-A1	EFNA1	1942	—3.2	—	—	—	—4.9	—16	—
Hydroxyprostaglandin dehydrogenase 15-(NAD)	HPGD	3248	—6.7	—	—	—	—	14	—
Isocitrate dehydrogenase 1 (NADP ⁺), soluble	IDH1	3417	—4.0	—	—5.3	—	—	3	—
Proenkephalin	PENK	5179	—38.0	6.4	13.9	25	—	—	—
Serum/glucocorticoid-regulated kinase	SGK	6446	—2.1	—	—4.9	—	—3.6	—	—
Transglutaminase 2	TGM2	7052	—4.4	—	—	—	—	—7	—
Tissue inhibitor of metalloproteinase 2	TIMP2	7077	—5.5	—	—	2.7	—	—	—

Gene name, gene ID derived from Entrez Gene; on, no expression detectable at oestrus or dioestrus respectively; e, expression detected; ¹ expression ratios between oestrus and dioestrus;² Ace et al. 2004 (rhesus monkey): expression ratios between sexual cycle day 13 and days 21–23; ³ Borthwick et al. 2003 (human): sexual cycle days 9–11 and 6–8 days after LH surge;⁴ Carson et al. 2002 (human): 2–4 days and 7–9 days after LH surge; ⁵ Kao et al. 2002 (human): sexual cycle days 8–10 and 8–10 days after LH surge; ⁶ Riesewijk et al. 2003 (human): 2 days and 7 days after LH surge; ⁷ Tan et al. 2003 (mouse): oestrus and dioestrus

signalling. The identification of this considerable number of genes involved in or regulated by TGF- β signalling is in line with current knowledge of regulation of the endometrium by TGF- β during the oestrous cycle, implantation and pregnancy (Godkin & Dore 1998, Tabibzadeh 2002). Furthermore, the induction of TGF- β mRNAs and proteins by oestrogen has been shown in epithelial cells of the mouse endometrium (Nelson *et al.* 1992). This fits very well with the results of our study, where at late oestrus (oestrogen levels low) target genes of TGF- β are still upregulated but negative regulators of this pathway are already present. Another pathway that could play a role in the regulation of the endometrial differentiation state is the RA signalling. In humans the regulatory role for endometrial maturation of RA signalling together with P₄ and TGF- β has already been described (Osteen *et al.* 2003). At dioestrus two mRNAs (*CYP26A1*, *RDHE2*) are elevated for proteins involved in RA metabolism whereas at oestrus the expression of two genes, which are described in context with differentiation was higher. Furthermore, the upregulation of mRNAs coding for proteins participating in the prostaglandin metabolism is consistent with the literature (Goff 2004, Spencer *et al.* 2004).

The comparison of the identified genes with microarray studies in other species revealed an overlap of 29%, whereas half of the changes in mRNA concentrations for these genes were not in the same direction. For the interpretation of this result it has to be considered that the overlap between the human studies was also relatively small for different reasons (see Riesewijk *et al.* 2003) although the same microarray platform (Affymetrix) was used. Furthermore, there are substantial differences between species regarding (i) endometrial changes during the cycle (oestrous cycle lesser changes than during menstrual cycle) and (ii) the type of implantation of the embryo. In humans there is an early (at the blastocyst stage) and invasive implantation, whereas in the bovine species implantation is less invasive and follows an extended peri-implantation period (after day 18 of the cycle) when the trophoblast spans nearly the complete uterus. In this context the comparison of these different gene expression profiles shows that there are common regulations but also distinct differences between species.

This is further supported by the expression patterns and/or potential functions that were already described for a number of the identified genes. Most of the data in the literature, although mainly derived from studies in other species, were in line with the results of the present study. Cycle-dependent expression with a higher level in proliferative in comparison with secretory phase endometrium was shown for example for *CTSK* (cathepsin K) (Jokimaa *et al.* 2001), *PRSS11* (serine protease 11, insulin-like growth factor (IGF) binding) (De

Luca *et al.* 2003), *PTH1H* (parathyroid hormone-like hormone) (Hoshi *et al.* 2001), *GJA1* (connexin 43) (Jahn *et al.* 1995), and *TNC* (tenascin C) mRNAs (Taguchi *et al.* 1999) in humans, and *SPARC* (osteonectin), *COL5A2*, *COL6A3*, *LGALS1* (galectin 1) and *SOX4* mRNAs in the Rhesus monkey (Ace & Okulicz 2004). In sheep, *GRP* (gastrin-releasing peptide) mRNA (Whitley *et al.* 1998) and *IGFBP6* (IGF-binding protein-6) mRNA (Gadd *et al.* 2002) showed maximal concentrations in the endometrium around ovulation. In the present study of bovine endometrium all these genes revealed higher expression levels at oestrus than at dioestrus. For two of these genes induction of mRNA expression by oestrogen has been shown in the endometrium of the rat for *GJA1* (Grummer *et al.* 1994) and in human endometrial stromal cells for *PTH1H* (Casey *et al.* 1993). *UTMP* mRNA expression in sheep endometrium was detected at day 15 of the oestrous cycle (Stewart *et al.* 2000) but at oestrus *UTMP* expression was not analysed. Here we show highest expression of *UTMP* mRNA in the bovine endometrium of the cranial uterine horns at oestrus.

Increased endometrial mRNA levels during the secretory phase were described for the RA catabolic enzyme *CYP26A1* (RA 4-hydroxylase), for *TGM2* (tissue transglutaminase) (Deng *et al.* 2003), and for *HPGD* (15-hydroxyprostaglandin dehydrogenase) (Kelly *et al.* 1994) in humans and the *PENK* mRNA in the rat (Jin *et al.* 1988). In mouse endometrium, positive regulation of *PENK* by P₄ has been indicated by a study of Cheon *et al.* (2002). In contrast to these findings, *PENK* is upregulated during proliferative phase in the endometrium of primates including humans (Low *et al.* 1989, Carson *et al.* 2002, Riesewijk *et al.* 2003, Ace & Okulicz 2004). *HPGD* and *TGM2* were also described to be upregulated by P₄ (Fujimoto *et al.* 1996, Greenland *et al.* 2000). In sheep, *TIMP2* (tissue inhibitor of metalloproteinase 2) mRNA (1.0 kb transcript) abundance in the endometrium has been shown to be stimulated by P₄ and to be increased at day 10 of the cycle (Hampton *et al.* 1995). All these genes showed higher expression levels at dioestrus compared with oestrus in the bovine endometrium.

Furthermore, a role during pregnancy or the implantation process was shown or assumed for *APOE* (apolipoprotein E) (Overbergh *et al.* 1995), *CLDN10* (claudin 10) (Wang *et al.* 2004), *EFNA1* (ephrin A1) (Fujiwara *et al.* 2002), *FBLN1* (fibulin 1) (Haendler *et al.* 2004), fibronectin (Rider *et al.* 1992), *GJA1* (Gabriel *et al.* 2004), *LGALS1* (Maquoi *et al.* 1997), *MGP* (matrix gla protein) (Spencer *et al.* 1999), *MMP2* (matrix metalloproteinase 2) (Goffin *et al.* 2003), *PENK* (Jin *et al.* 1988), *PLA2 G10* (phospholipase A2) (Goff 2004), *SERPINA1* (Marshall & Braye 1987), *TNC* (Michie & Head 1994) and *UTMP* (Hansen 1998).

The endometrium is a relatively complex tissue composed of many different cell types. For the

interpretation of differential gene expression the assignment to distinct cell types is important. Therefore, *in situ* hybridisations for five selected genes were done. All five genes were expressed specifically in the endometrium, mainly in the surface and the glandular epithelium. Nevertheless, there were distinct differences in their expression patterns, for example *INHBA* and *PTHLH* mRNAs were also detectable in fibroblasts of the stroma. The expression pattern of the *UTMP* mRNA in the bovine uterus at oestrus was similar to that found in sheep at day 19 of pregnancy (Stewart *et al.* 2000). For *PENK* the mRNA expression was highest in the deep glandular similar to the localisation in the mouse uterus (Rosen *et al.* 1990).

Our study is the first holistic screen for transcriptome changes in bovine endometrium between oestrus and dioestrus. We identified a number of molecular pathways, most prominently the TGF- β pathway, involved in the marked functional changes of endometrium between these two stages. To complete the analysis of gene expression during the oestrus cycle further stages (e.g. prooestrus and metoestrus) will be investigated and the function of candidate genes, e.g. *UTMP*, will be characterised. Furthermore, the differentially regulated genes identified in this study represent an important basis for future studies, e.g. on embryo–maternal interactions during the pre-implantation period, and for differential diagnosis of fertility problems via array-based analysis of endometrial biopsies.

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