Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle

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Abstract

The oviduct epithelium undergoes marked morphological and functional changes during the oestrous cycle. To study these changes at the level of the transcriptome we did a systematic gene expression analysis of bovine oviduct epithelial cells at oestrus and dioestrus using a combination of subtracted cDNA libraries and cDNA array hybridisation. A total of 3072 cDNA clones of two subtracted libraries were analysed by array hybridisation with cDNA probes derived from six cyclic heifers, three of them slaughtered at oestrus and three at dioestrus. Sequencing of cDNAs showing significant differences in their expression levels revealed 77 different cDNAs. Thirty-seven were expressed at a higher level at oestrus, for the other 40 genes expression levels were higher at dioestrus. The identified genes represented a variety of functional classes. During oestrus especially genes involved in the regulation of protein secretion and protein modification, and mRNAs of secreted proteins, were up-regulated, whereas during dioestrus particularly transcripts of genes involved in transcription regulation showed a slight up-regulation. The concentrations of seven selected transcripts were quantified by real-time RT-PCR to validate the cDNA array hybridisation data. For all seven transcripts, RT-PCR results were in excellent correlation ($r > 0.92$) with the results obtained by array hybridisation. Our study is the first to analyse changes in gene expression profiles of bovine oviduct epithelial cells during different stages of the oestrous cycle, providing a starting point for the clarification of the key transcriptome changes in these cells.

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Introduction

The oviduct epithelium is important for different reproductive processes and undergoes marked morphological and functional changes during the oestrous cycle. It has been shown that a dramatic change in the frequencies of ciliated and non-ciliated cells occurs during the oestrous cycle (Yaniz *et al.* 2000). At pre-oestrus the epithelium consists mainly of secretory cells and at dioestrus of ciliated cells. A recent study implies that the individual epithelial cells change their functional status, rather than the whole cell population being renewed, during the oestrous cycle (Suuroia *et al.* 2002).

The oviduct is responsible for the transport of ova, sperm and embryos, and the oviduct epithelial cells are the first to have contact with the ova and the cleavage-stage embryos. The isthmus part is described as functioning as a sperm reservoir – sperm adhere to the epithelium and at the time of ovulation they are released (Hunter & Wilmut 1984) to ensure the perfect timing of fertilisation. In this way, the oviduct provides the microenvironment for sperm capacitation, fertilisation, and early embryonic development (Buhi 2002).

At the molecular level only few genes or proteins are known that change in activity or abundance during the oestrous cycle and may be important for

fertility, such as the bovine oviduct-specific glycoprotein, the major secretory protein in the oviduct (Boice *et al.* 1990). This protein has been described as having a positive influence on sperm capacitation and motility, the interaction of the gametes and fertilisation (for a review, see Buhi 2002). The expression of this glycoprotein is oestrogen-dependent with the highest level at oestrus and the lowest in the luteal phase. However, the production of a null mutation of the oviduct-specific glycoprotein (OVGP1) gene in a recent study in the mouse showed that fertility of *ovgp1*^{-/-} females was in normal limits, indicating that OVGP1 is not essential for fertilisation (Araki *et al.* 2003).

Recently, we did a first systematic analysis of gene expression in bovine oviduct epithelial cells at day 3.5 of the oestrous cycle comparing the ipsilateral and the contralateral oviduct (Bauersachs *et al.* 2003). For that study we successfully used a combination of subtracted cDNA libraries and cDNA array hybridisation to identify cDNAs differentially regulated in the ipsi- vs the contralateral oviduct. In the present study we investigated the mRNA expression profile in epithelial cells of the ipsilateral oviduct at oestrus and dioestrus to provide a starting point for the identification of key transcriptome changes during the oestrous cycle. The results obtained by cDNA array hybridisation were verified for seven selected transcripts by absolute quantification using a real-time RT-PCR approach.

Materials and methods

Synchronisation of oestrous cycle and isolation of oviduct epithelial cells

Six cyclic heifers (Deutsches Fleckvieh) between 22 and 36 months old were treated with a progesterone (P₄)-releasing intravaginal device (1.94 g P₄) (EAZI-Breed CIDR; Animal Reproductive Technologies Ltd, Leominster, Hereford and Worcs, UK) for 11 days beginning at dioestrus. After removal a single dose of 500 µg cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) was administered i.m. Animals were observed for sexual behaviour (i.e. toleration, sweating, vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate injection. Three animals (group A) were slaughtered the morning

after standing heat occurred and three animals (group B) 12 days after oestrus. Blood samples were taken just before slaughtering to determine P₄ serum levels. Group A animals displayed low serum P₄ levels (< 3.0 ng/ml) and group B animals had high serum P₄ levels (> 12.0 ng/ml). Oviducts were trimmed free of surrounding tissue and ligated at the infundibulum and at the isthmus-uterus junction. The complete organs were rinsed with PBS. After opening the oviduct longitudinally, epithelial cells were isolated by scraping the mucosal epithelial layer with a sterile glass slide (Reischl *et al.* 1999). Within 10 min after death cells were transferred into cryotubes and immediately dropped into liquid nitrogen for transport, and were stored at -80 °C until further processing. All experiments with animals were carried out with permission from the local veterinary authorities.

Oligonucleotides for suppression subtractive hybridisation (SSH)

The following oligonucleotides were used for SSH: cDNA primer 1: AACTGCGGCCGCGTA CAGCT₂₀VN (V=A, C or G), cDNA primer 2: GAGAT₂₀VN, adapter 1: 5'-GTAATACGACTC ACTATAGGGCTCGAGCGCGCCGCGAGGGC AGTG-3', adapter 1 reverse: 5'-CACTGCCCTG CGGG-3', adapter 2: 5'-GTAATACGACTCAC TATAGGGCAGGGCGTGTTGCGCGCTGC TGG-3', adapter 2 reverse: 5'-CCAGCAGCGC GCAG-3', PCR primer 1: 5'-GTAATACGACT CACTATAGGGC-3', nested primer 1: 5'-TCG AGCGCGCCGCGAGGGCAGTG-3', and nested primer 2: 5'-AGGGCGTGGTGC GCGCTGC TGG-3'.

Generation of subtracted cDNA libraries

The production of subtracted libraries was done according to the SSH method (Diatchenko *et al.* 1996, 1999, Gurskaya *et al.* 1996). Total RNA from bovine oviduct epithelial cells was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. For each stage of the cycle, oestrus and dioestrus, RNA of two animals was pooled. Double-stranded (ds) cDNA was synthesised starting with 80 µg total RNA (corresponding to approximately 1.5–2 µg mRNA) using Superscript II (400 U) (Invitrogen) and cDNA primer 1 (50 pmol) for first-strand synthesis. The second

strand was synthesised with *Escherichia coli* DNA polymerase I (40 U), *E. coli* RNase H (2 U), and *E. coli* DNA ligase (10 U) (Invitrogen) according to the manufacturer's instructions. Ribosomal and other residual RNAs were digested with 3 µl RNase (0.5 mg/µl, DNase-free, from bovine pancreas; Roche Diagnostics) for 90 min at 37 °C. cDNA was then incubated with 10 U T4 DNA polymerase (Roche) (5 min at 16 °C) to yield blunt ends. After phenol/chloroform extraction and DNA precipitation cDNA was digested with RsaI (Roche). Tester cDNA was ligated with adapter 1/1 rev or adapter 2/2 rev overnight at 16 °C using T4 DNA ligase (400 U; New England Biolabs). The first step of SSH was performed in 4 µl for 8 h and the second hybridisation in 9 µl for 20 h at 68 °C. Excess of driver was 30-fold in the first step of SSH. Suppression PCR was performed with the following parameters for primary PCR reactions: 75 °C for 5 min; 96 °C for 60 s; 29 cycles (dioestrus) or 31 cycles (oestrus) at (94 °C for 30 s, 66 °C for 30 s, 72 °C for 1.5 min); 72 °C for 3 min. PCR products were diluted 10-fold and 1 µl of the dilution was used as a template for secondary PCR. This reaction was performed using nested primers with the following parameters: 96 °C for 1 min; 12 cycles (dioestrus) or 14 cycles (oestrus) at (94 °C for 30 s, 68 °C for 30 s, 72 °C for 1.5 min); 72 °C for 3 min. All PCR reactions were done in a Perkin Elmer Cetus thermal cycler using the Advantage 2 Polymerase Mix (BD Clontech, Heidelberg, Germany). After phenol/chloroform extraction and DNA precipitation PCR products were digested with BssHII (New England Biolabs) (inside the adapter sequences), purified by agarose gel electrophoresis, and ligated overnight with the AscI-digested (New England Biolabs) and dephosphorylated vector DNA (derivative of pBSIISK[−]; Stratagene). The ligation reaction was introduced directly into *E. coli* SURE (Stratagene) by electroporation. The subtracted libraries were stored as glycerol stocks at −80 °C.

Preparation of cDNA arrays

cDNA array hybridisation was done essentially as previously described (Bauersachs *et al.* 2003). In brief, 1536 cDNA clones were randomly picked for each library and grown overnight at 37 °C in 100 µl LB medium in 96-well microtitre plates. Bacterial suspensions were diluted 20-fold in

Tris/HCl-EDTA(TE) buffer (pH 8.0), incubated at 96 °C for 10 min, and stored at −20 °C. PCR amplification of the cDNA insertions was performed in 20 µl in 96-well cycle plates and contained 1 µl diluted bacterial cells, PCR buffer (peQLab, Erlangen, Germany), 200 pmol/µl dNTPs, 0.3 pmol/µl of the respective primers (modified T3 and T7 primers), and 1 U Taq DNA polymerase (peQLab). All PCR products were analysed by 0.8% agarose gel electrophoresis showing that more than 95% of the PCR reactions yielded defined PCR products. Fifteen microlitres of PCR reactions were transferred to 384-well microtitre plates (Nunc) containing 15 µl 2-fold spotting buffer (40 mM Tris-HCl pH 8, 2 M NaCl, 2 mM EDTA, bromophenol blue). A total of 1536 PCR products were spotted onto nylon membranes (Nytran Supercharge; Schleicher & Schuell, Dassel, Germany) on an area of 20 × 50 mm using an Omnigrid Accent microarrayer (GeneMachines, San Carlos, CA, USA) and solid pins (SSP015, diameter 0.015 inches; Telechem International, Sunnyvale, CA, USA). Spotting was done six times for each PCR product on the same position for sufficient and equal application; 25 arrays were produced simultaneously. The overall 3072 PCR products had to be distributed on two arrays that contained 1536 cDNAs (768 cDNAs of each subtracted library per array). The spotted cDNAs were denatured on the arrays by incubation on filter paper (Schleicher & Schuell) soaked with 0.5 M NaOH for 20 min at room temperature. DNA was fixed by baking at 80 °C for 30 min and UV cross-linking (120 mJ/cm²) (XL-1500 UV Crosslinker; Spectronics Corp., New York, USA).

cDNA array hybridisation

RNA of all six animals (oestrus *n*=3, dioestrus *n*=3) was converted to ds cDNA essentially as described above but using cDNA primer 2. ³³P-labelled cDNA probes were generated from ds cDNA corresponding to 7.5–15 µg total RNA. cDNA was heat-denatured for 10 min at 96 °C and then chilled on ice. High Prime reaction mixture (Roche), dNTP mixture (dCTP final concentration 10 pmol/µl, dATP, dGTP, and dTTP final concentration each 100 pmol/µl) and 90 µCi [α-³³P]dCTP were added to a final volume of 20 µl. Reactions were incubated for 4 h at 37 °C and subsequently purified with MicroSpin G-25 Columns (Amersham Biosciences)

to remove unincorporated nucleotides and to estimate labelling efficiency. Hybridisation was done as follows: pre-hybridisation was done for up to six arrays together in one 15 cm glass hybridisation bottle: 3×10 min 10 ml $1 \times$ PBS/10% SDS at 65 °C; 2×10 min 10 ml $0.1 \times$ PBS/1% SDS at 85 °C; 3×10 min 10 ml $1 \times$ PBS/10% SDS at 65 °C. Hybridisation probes were denatured for 15 min at 96 °C immediately before adding to the hybridisation solution. Hybridisation was done in plastic vials (Poly-Q vials, 18 ml; Beckman Coulter, Munich, Germany) in 2 ml $1 \times$ PBS, pH 7.5/10% SDS for 45 h at 65 °C. After hybridisation, arrays were put together in one 15 cm glass hybridisation bottle and washed as follows: 3×5 min 10 ml $1 \times$ PBS/10% SDS at 65 °C; 3×10 min 10 ml $1 \times$ PBS/10% SDS at 65 °C; 3×10 min 10 ml $0.1 \times$ PBS/1% SDS at 65 °C and finally 2×5 min 10 ml $1 \times$ PBS/1% SDS/2 mM EDTA at room temperature. Filters were dried by baking at 80 °C for 20 min and exposed to an imaging plate BAS-SR (Fuji Photo Film Co.) Imaging plates were scanned with a phosphor imager (Storm 860; Amersham Biosciences).

To facilitate optimal evaluation of the hybridisation signals, 212 of the 3072 cDNA fragments were removed from the arrays due to their very high signal intensity that interfered with the analysis of approximately 20% of all signals; 103 cDNA fragments were from the oestrous library and 109 from the dioestrous library. Hybridisation of the arrays with the cDNA for bovine mRNA for 95 kDa OVGP1 showed that approximately 70 cDNA fragments corresponded to the OVGP1 cDNA. The OVGP1-positive cDNA fragments came from the oestrous library and showed higher expression at oestrus. The cDNAs that were removed in addition to the OVGP1 cDNAs showed no differential signals between oestrus and dioestrus.

These optimised arrays were used for the final hybridisation analysis as described above.

Analysis of array data

Array evaluation was done using AIDA Image Analyzer (Version 3.27; Raytest, Straubenhardt, Germany). Background was subtracted with the 'Weighted background dot' function. Raw data obtained by AIDA Array software were exported to Microsoft Excel and normalised to the median of

each array. These normalised values of the six hybridisation experiments were then used for statistical analysis (Student's *t*-test). Differences were considered significant at $P < 0.05$ and a ratio ≥ 1.5 . Reproducibility of the used array system was shown previously (Bauersachs *et al.* 2003).

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 Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi>). cDNAs without similar entries in the 'nr' database (all GenBank+RefSeq Nucleotides+EMBL+DDBJ+PDB sequences, but no EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) were in addition compared with themselves to find redundant cDNA fragments. Based on the human or mouse homologues simplified Gene Ontologies were built using GeneSpring software version 6.1 (Silicon Genetics, Redwood City, CA, USA). Data from other sources, for example UniGene (www.ncbi.nlm.nih.gov/uniGene/), LocusLink (www.ncbi.nlm.nih.gov/locusLink/), and PubMed (www.ncbi.nlm.nih.gov/entrez/query.fcgi), were also integrated. The obtained sequences were submitted to dbEST (a division of GenBank).

Primer pairs for real-time RT-PCR

The following primers were used to amplify specific fragments referring to selected regulated genes (for abbreviations see Table 1, length of PCR products in square brackets): TRAI (for 5'-TGGCAGAG ACCATCGAAAG-3'; rev 5'-GGTAACTTCCC CTTTCAGCAG-3' [120 bp]), ERP70 (for 5'-TCGA CTACATGATGGAGCAG-3'; rev 5'-CCGACT TAAAGACTCCGATG-3' [117 bp]), GRP78/HSPA5 (for 5'-AACGACCCCTGACGAAAG AC-3'; rev 5'-TCACTCGAAGAATGCCATTC AC-3' [129 bp]), AGR2 (for 5'-AACTCAAAC TGCCCCAGACC-3'; rev 5'-ACAAACTGCTC TGCCAATCTC-3' [205 bp]), OVGP1 (for 5'-TG TCCACGTTTTCCAACCG-3'; rev 5'-GGAGG

Table 1 Genes up-regulated at oestrus

	Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Oe:Di*	P value
Bovine cDNA/gene or homologue	FLJ37927; CDC20-like protein (FLJ37927)	NM_152623	Hs.170708	78	Cell cycle control	—	—	—	7.5	0.023
	<i>Homo sapiens</i> CDC28 protein kinase regulatory subunit 2	NM_001827	Hs.83758	93	Cell cycle control	Regulation of CDK activity; cell cycle; cytokinesis	Cyclin-dependent protein kinase activity	—	7.1	0.006
	<i>Homo sapiens</i> mRNA for KIAA1154 protein	AB032980	Hs.61345	78	Cellular defence response	Cellular defence response	Tumour antigen	—	1.9	<0.001
	<i>Homo sapiens</i> lymphocyte cytosolic protein 1 (L-plastin)	NM_002298	Hs.381099	91	Cytoskeleton; actin-bundling protein	—	Actin binding; calcium ion binding	Cytosol	2.6	0.048
	<i>Homo sapiens</i> retinoic acid receptor (tazarotene induced) 1	NM_002888	Hs.82547	84	Membrane receptor; cell adhesion molecule?	Negative regulation of cell proliferation	—	Integral to membrane	3.2	0.002
	<i>Homo sapiens</i> hyperpolarization-activated cyclic nucleotide-gated potassium channel 4	HCN4	NM_005477	Hs.225671	Muscle contraction; pacemaker channel	Microtubule-based movement; sodium ion transport; potassium ion transport; muscle contraction	Voltage-gated potassium channel activity; structural molecule activity; 3',5'-cAMP binding	Microtubule; integral to plasma membrane; membrane fraction	2.9	<0.001
	<i>Bos taurus</i> mRNA for putative endoplasmic reticulum nucleotide sugar transporter	SLC35B1; UGTREL1	Hs.154073	100	Nucleotide sugar transporter	Transport	UDP-galactose transporter activity	Microsome	2.3	0.017
	<i>Homo sapiens</i> arginine-rich, mutated in early stage tumours	ARMET; ARP	NM_006010	Hs.436446	Oncogenesis	—	—	—	4.0	0.006
	<i>Homo sapiens</i> stromal cell-derived factor 2-like 1	SDF2L1	NM_022044	Hs.303116	Protein modification?; stress protein; O-mannosyltransferase?	—	—	Membrane; endoplasmic reticulum	9.3	<0.001
	<i>Homo sapiens</i> protein disulphide isomerase related protein (calcium-binding protein, intestinal-related)	ERP70; ERP72	NM_004911	Hs.93659	Protein secretion; chaperone	Protein secretion	Protein disulphide isomerase activity	Endoplasmic reticulum lumen	12.3	0.005
	<i>Bos taurus</i> tumour rejection antigen (gp96) 1	TRA1; GP96; GRP94	NM_174700	Hs.192374	Protein secretion; chaperone; immune-related	Protein folding	Chaperone activity	Endoplasmic reticulum	11.4	<0.001
	<i>Homo sapiens</i> heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa)	HSPA5; BIP; MIF2; GRP78	NM_005347	Hs.310769	Protein secretion; chaperone	—	Hsp70/Hsp90 organising protein activity, ATP binding	Endoplasmic reticulum lumen	6.1	<0.001
	<i>Homo sapiens</i> hypoxia up-regulated 1	HYOU1; ORP150	NM_006389	Hs.277704	Protein secretion; chaperone	Response to stress	Chaperone activity	Endoplasmic reticulum	5.4	0.002

Table 1 Continued

Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Oe:Di*	P value
Bovine cDNA/gene or homologue									
<i>Homo sapiens</i> DNAJ (Hsp40) homologue subfamily B, member 11	DNAJB11; EDJ; HEDJ; ABBP2; ABBP-2	HS.317192	94	Protein secretion; chaperone	Protein folding	Chaperone activity	Endoplasmic reticulum	4.4	0.002
<i>Homo sapiens</i> protein disulphide isomerase-related protein	P5	HS.212102	91	Protein secretion; chaperone	Electron transport; protein folding	Protein disulphide isomerase activity	Endoplasmic reticulum	3.6	0.001
<i>Homo sapiens</i> endoplasmic reticulum degradation enhancing alpha mannosidase-like	EDEM; KIAA0212	HS.154332	91	Protein secretion; degradation of misfolded glycoproteins	N-linked glycosylation; carbohydrate metabolism	Mannosyl-oligosaccharide 1,2-alpha-mannosidase activity; calcium ion binding	Integral to membrane	3.3	<0.001
<i>Bos taurus</i> glucose regulated protein 58 kDa	GRP58; ERp57; ERp60; ERp61; GRP57; PI-PLC	HS.308709	99	Protein secretion; chaperone	Protein-nucleus import; protein-endoplasmic reticulum retention; signal transduction	Phospholipase C activity; protein disulphide isomerase activity; cysteine-type endopeptidase activity	Endoplasmic reticulum	3.1	0.005
<i>Homo sapiens</i> signal sequence receptor, delta (translocon-associated protein delta)	SSR4; TRAPD	HS.409223	87	Protein secretion	Intracellular protein transport	Calcium ion binding	Endoplasmic reticulum; integral to membrane; translocon	2.3	0.004
<i>Homo sapiens</i> anterior gradient 2 homologue (<i>Xenopus laevis</i>) Gastrin-releasing peptide (GRP) (sheep)	AGR2; AG2; GOB-4; HAG-2; XAG-2 GRP	HS.226391	80	Secreted protein; ECM protein; differentiation	—	—	Extracellular space	6.9	0.029
<i>Oryctolagus cuniculus</i> hensin	DMBT1; GP340	HS.279611	86	Secreted protein; hormone regulation; multifunctional	Neuropeptide signalling pathway; signal transduction	Growth factor activity	Soluble fraction	4.0	<0.001
<i>Homo sapiens</i> deleted in malignant brain tumours 1 (DMBT1)	NM_007329	—	79	Secreted protein; ECM protein	—	Tumour suppressor	—	3.1	<0.001
Bovine mRNA for 95 kDa oviduct-specific glycoprotein	OVGP1; MUC9	HS.1154	99	Secreted protein; fertility control	Pregnancy; fertilization	Hydrolase activity	Extracellular space	2.7	0.003
<i>Homo sapiens</i> sel-1 suppressor of lin-12-like	SEL1L; IBD2; SEL1-LIKE	HS.181300	91	Signal transduction?	—	—	Integral to membrane, extracellular space	3.7	0.003
Human gene for U3 small nuclear RNA	U3 snRNA	—	87	Small nuclear RNA	—	—	—	3.6	0.005
<i>Homo sapiens</i> chromosome 5 clone CTD-2124P24	—	AC079465	78	Unknown	—	—	—	18.4	0.005
1JEU21G7 <i>Bos taurus</i> Jejunum #1 library	—	CB223287	100	Unknown	—	—	—	5.8	0.006
<i>Bos taurus</i> cDNA	—	—	—	Unknown	—	—	—	—	—
<i>Mus musculus</i> adult male corpus striatum cDNA	C030027L06Rik	Mm.71000	83	Unknown	—	—	—	5.0	0.022

Table 1 Continued

Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Oe:Di*	P value
Bovine cDNA/gene or homologue									
Human DNA sequence from clone RP11-244N9 on chromosome 9	AL162385		80	Unknown	—	—	—	4.8	0.021
<i>Homo sapiens</i> hypothetical protein FLJ10707; KIAA1757	NM_018187	Hs.405917	96	Unknown	—	—	—	4.7	<0.001
<i>Homo sapiens</i> similar to RIKEN cDNA 1700018018 gene	BC011587	Hs.75668	90	Unknown	—	—	—	2.3	0.007
528733 MARC 3BOV	BM287908	—	97	Unknown	—	—	—	2.2	0.004
<i>Bos taurus</i> cDNA	BE665083	—	100	Unknown	—	—	—	1.9	0.004
153052 MARC 4BOV	—	—	—	Unknown	—	—	—	—	—
<i>Bos taurus</i> cDNA 5 cDNA fragments without homology to database sequences	—	—	—	Unknown	—	—	—	—	—

*Mean oestrus:mean dioestrus.
ECM, extracellular matrix.

GCGATCACTGAACTG-3' [856 bp]) referring to Rief *et al.* (2002), C3 (for 5'-AAGTTCATCAGCC ACATCAAG-3'; rev 5'-CACTGTTTCTGGTTC TCCTC-3' [191 bp]), MS4A8B (for 5'-CTGAC CTGCTACCAAACCAAC-3'; rev 5'-GCCCA AACAAGGGAGGTATTC-3' [191 bp]) and 18S rRNA (for 5'-AAGTCTTTGGGTTCCG GG-3'; rev 5'-GGACA TCTAAGGGCATC ACA-3' [365 bp]) referring to Schoenfelder & Einspanier (2003).

Reverse transcription and real-time RT-PCR

One microgram of each sample of total RNA (oestrus $n=3$, dioestrus $n=3$) was reverse transcribed in a total volume of 60 μ l: 5 $\times$ buffer (Promega), 10 mM dNTPs (Roche), 50 μ M hexamer primers (Gibco BRL), 200 U Superscript RT enzyme (Promega). The conventional PCRs were performed in a thermal cycler (Biometra, Göttingen, Germany) as previously described (Schams *et al.* 2002). All amplified PCR fragments were commercially sequenced to verify the resulting PCR product (MWG-Biotech, Ebersberg, Germany). Thereafter the specific melting point (MP) of the amplified product carried out within the LightCycler standard PCR protocol served as verification of the product identity (Pfaffl *et al.* 2003). The obtained sequences were subsequently submitted to the EMBL database. For each of the following real-time PCR reactions, 1 μ l 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 (Einspanier *et al.* 2002). In each PCR reaction 17 ng/ μ l cDNA were introduced and amplified in a 10 μ l reaction mixture (3 mM MgCl₂, 0.4 μ M primer forward and reverse each, 1 $\times$ LightCycler DNA Master SYBR Green I (Roche)). The annealing temperature was 60 °C for all PCRs. The MP and the appropriate fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment were as follows: TRA1 (MP 81 °C, FA 77 °C), ERP70 (MP 88 °C, FA 84 °C), GRP78/HSPA5 (MP 85 °C, FA 81 °C), AGR2 (MP 83 °C, FA 77 °C), OVGP1 (MP 89 °C, FA 85 °C), C3 (MP 89 °C, FA 85 °C), MS4A8B (MP 85 °C, FA 81 °C), and 18S rRNA (MP 88 °C, FA 82 °C).

Data analysis of real-time RT-PCR

The normal distribution of the data was tested by the Kolmogorow–Smirnov method. The significance of differences was evaluated by a *t*-test (Sigma Stat, version 2.03). For all quantitative assays a calibration curve based on a purified PCR product was used. The efficiencies of the specific PCRs were determined as described by Schoenfelder & Einspanier (2003). Correlation of real-time RT-PCR and cDNA array hybridisation results was calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com).

Results

To analyse the reflections of the morphological changes in the bovine oviduct epithelium on the transcriptional activity we used a combination of subtracted cDNA libraries and cDNA array hybridisation. Two subtracted cDNA libraries were produced for oestrus and dioestrus respectively; 1536 randomly picked cDNA clones of each library were analysed by cDNA array hybridisation. The first array hybridisations showed that many cDNAs were on the array, which produced very strong signals (Fig. 1a) and interfered with the analysis of up to 20% of all signals. A hybridisation of the arrays with a cDNA probe for the bovine mRNA for 95 kDa OVGP1, which was known to be expressed at very high levels especially at oestrus, showed that approximately 70 of the 3072 cDNAs corresponded to this gene (Fig. 1b). After removal of the OVGP1 cDNA fragments except for one and an additional 142 cDNA fragments that also revealed very strong signals (and no differential expression) the hybridisation signals were much easier to analyse (Fig. 1c). These optimised cDNA arrays were then hybridised with probes of six cyclic heifers (oestrus $n=3$, dioestrus $n=3$).

The data analysis revealed 235 cDNA fragments showing significant signal differences between oestrus and dioestrus. In Figure 2a a scatter plot of the array data for these cDNA fragments is shown. This illustration shows that more cDNA fragments were found in the subtracted libraries, which were up-regulated at oestrus. However, the sequence analyses of these 235 cDNA fragments revealed 37 different genes with higher expression levels at oestrus and 40 with increased expression at

Figure 1 Removal of cDNA fragments of highly abundant mRNAs. cDNA array hybridisation yielded for a number of cDNA clones very strong signals, which interfered with the analysis of up to 20% of all signals (a). To show that many of these cDNAs correspond to the bovine mRNA for 95 kDa OVGP1, arrays were hybridised with a cDNA probe for OVGP1 (b). All but one cDNA for this gene and 142 additional cDNAs, which yielded very strong signals (and no differential expression), were removed from the arrays and cDNA array hybridisation was repeated using this optimised array (c).

dioestrus (Fig. 2b). The average signal ratio was 4.8 for the genes up-regulated at oestrus and 2.2 for those up-regulated at dioestrus. Twenty-one genes were found more than once, most frequently the *Bos taurus* tumour rejection antigen (gp96) 1 (TRA1) ($n=56$), the bovine homologue to the *Homo sapiens* anterior gradient 2 homologue (*Xenopus laevis*) (AGR2) ($n=24$) and the *Homo sapiens* heat shock 70 kDa protein 5 (glucose-regulated protein, 78 kDa) (HSPA5) ($n=16$). For genes that were represented with multiple copies on the array the ratios for the optimal quantifiable signals are shown in the tables rather than the mean of all signal ratios. Nevertheless, the fold differences of the intra-array replicates compared very well. Also, the expression changes revealed by different fragments of one cDNA were quite similar, as observed previously (Bauersachs *et al.* 2003).

Table 1 lists the cDNAs whose corresponding mRNAs were more abundant in the oviduct epithelial cells at oestrus. The cDNAs are sorted

alphabetically by their probable protein function and their signal ratio (descending). The common name, the GenBank accession number, the corresponding UniGene entry, the percent homology to the database sequence, the Gene Ontologies, and the *P* values are also shown. The signal ratios oestrus:dioestrus were up to 18-fold. Twenty-four of the cDNAs were assigned to genes whose function is known or inferred, mostly to the probable human homologue. For five cDNAs no hit in the GenBank database ('nr' and 'est') was found. In Table 2 the results are shown for the mRNAs, which were more abundant in the oviduct epithelial cells at dioestrus. The greatest difference in gene expression between dioestrus:oestrus was 4.4-fold. Twenty-two of the 40 cDNAs corresponded to genes whose function is known or inferred. Two cDNAs could not be assigned to any sequence in the GenBank database.

The results of the cDNA array hybridisation were verified and absolutely quantified by the use

Figure 2 (a) Scatter plot of all differentially expressed cDNA fragments. The mean signals at oestrus (vertical axis) were plotted against the mean signals at dioestrus (horizontal axis) for all 235 differentially expressed cDNA fragments. The lines mark 1.5-fold difference in gene expression. (b) Overview of the identified differentially expressed genes and their assignment to different functional categories.

of real-time RT-PCR. The same six RNA samples (oestrus $n=3$, dioestrus $n=3$) as for array hybridisation were used. As standardisation for the gene-specific data generated from the real-time specific approach, the abundance of the 18S ribosomal RNA was used that was not different between samples of oestrus and dioestrus (see Table 3). Since we referred the absolute mRNA amounts to the amount of total RNA, similar values for the 18S rRNA indicate that similar amounts of total RNA were used for the cDNA synthesis. All seven selected genes that had been found up- or down-regulated at oestrus revealed significant differences in the mRNA quantification approach compared with dioestrus. The mean concentrations of pg mRNA/ μ g total RNA $\pm$ S.E.M., the mean oestrus:dioestrus gene expression differences, and the significance values are provided in Table 3. The mRNA concentrations ranged from 2 pg (AGR2) to 371 pg (OVGP1) per μ g total RNA at oestrus. The corresponding concentrations at dioestrus were 0.2 and 70 pg/ μ g total RNA respectively. Out of the group of selected genes up-regulated at oestrus, the 19-fold increase of TRA1 was highest, whereas a 1.7-fold up-regulation of MS4A8B at

dioestrus could be detected representing the least but still significant change of expression. For all transcripts investigated there was a strong correlation between cDNA array hybridisation and real-time RT-PCR data, as illustrated in Fig. 3.

To get a more detailed view of the differentially expressed genes regarding their assignment to functional classes a simplified Gene Ontology for the biological processes and molecular functions was built (Fig. 4). Some of the biological processes and molecular functions were clearly dominated by genes up-regulated either at oestrus or at dioestrus. For example, the biological process 'Protein secretion' was clearly up-regulated at oestrus indicated by seven chaperones and two other proteins located in the endoplasmic reticulum (ER). In contrast, at dioestrus genes involved in transcriptional regulation and genes with immune-related functions were up-regulated. For other biological processes and molecular functions shown in Fig. 4 the mRNA abundance of identified genes was higher at oestrus or at dioestrus. Further analysis of these groups of genes showed that they belong to different functional subclasses (see Tables 1 and 2).

Table 2 Genes up-regulated at dioestrus

	Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Di:Oe* P value
Bovine cDNA/gene or homologue									
<i>Homo sapiens</i> cyclin D1 (PRAD1; parathyroid adenomatosis 1)	CCND1; BCL1; PRAD1; U21B31; D11S287E	NM_053056	Hs.371468	81	Cell cycle regulation	G1/S transition of mitotic cell cycle; cell growth and/or maintenance; cytokinesis	—	Nucleus	2.9 <0.001
<i>Homo sapiens</i> nucleoside phosphorylase	NP; PNP	NM_000270	Hs.75514	84	Enzyme, purine synthesis	DNA modification; nucleobase, nucleoside, nucleotide and nucleic acid metabolism	Purine-nucleoside phosphorylase activity; transferase activity, transferring glycosyl groups	—	2.6 0.018
<i>Bos taurus</i> mRNA for similar to NADH dehydrogenase subunit 1	MTND1	AB098969	—	98	Enzyme, energy metabolism	Mitochondrial electron transport, NADH to ubiquinone	NADH dehydrogenase (ubiquinone) activity; oxidoreductase activity	Respiratory chain complex 1; mitochondrion; integral to membrane	1.8 0.009
<i>Homo sapiens</i> complement component 3	C3; ASP	NM_000064	Hs.284394	82	Immune response, complement activity	Complement activation, classic pathway, alternative pathway; G-PCR protein signalling pathway; immune response	Complement activity; endopeptidase inhibitor activity; receptor binding	Extracellular	4.0 <0.001
<i>Mus musculus</i> FK506 binding protein 1a	FKBP1A; PKC12; PKC12; FKBP12; PPIASE; FKBP-12; FKBP12C	NM_008019	Hs.374638	92	Immune-related protein folding, signalling	Protein folding	Receptor activity; FK506 binding; FK506-sensitive peptidyl-prolyl cis-trans isomerase; cyclophilin-type peptidyl-prolyl cis-trans isomerase activity	—	2.6 <0.001
<i>Homo sapiens</i> 5'-nucleotidase, ecto	NT5E; eN; NTE; eNT; CD73; E5NT	NM_002526	Hs.153952	79	Immune-related, anti-inflammatory, signalling	DNA metabolism; nucleotide catabolism	Hydrolase activity, acting on ester bonds; IMP-GMP specific 5'-nucleotidase	Membrane fraction	1.7 0.017
<i>Homo sapiens</i> pyruvate dehydrogenase kinase 4 mRNA	PKD4	AF334710	Hs.8364	85	Kinase	Glucose metabolism; signal transduction	Pyruvate dehydrogenase (lipoamide) kinase activity; ATP binding; protein kinase activity; transferase activity	Mitochondrion	1.8 <0.001
598768 MARC 6BOV <i>Bos taurus</i> cDNA 3' <i>Homo sapiens</i> SMT3 suppressor of mit two 3 homologue 1 (yeast), mRNA	SMT3H1; SMT3A; SUMO-3	CB24507	Hs.85119	100	Kinetochore regulation	—	—	Kinetochore	1.9 0.019
<i>Homo sapiens</i> membrane-spanning 4-domains, subfamily A, member 8B	MS4A8B; MS4A4; 4SPAN4	NM_031457	Hs.150184	82	Membrane receptor, signalling	Transport	Transporter activity; receptor activity	Integral to membrane	2.1 0.001
<i>Homo sapiens</i> O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase)	OGT; HRNT1; FLJ23071; MG22921; O-GLCNAC	NM_003605	—	90	Protein modification, signalling	Response to nutrients; O-linked glycosylation; signal transduction	Acetylglucosaminyl-transferase activity; protein binding; transferring glycosyl groups	Cytosol; nucleus	1.8 <0.001

Table 2 Continued

Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Di:Oe*	P value
Bovine cDNA/gene or homologue									
<i>Homo sapiens</i> ribosomal protein L5 (RPL5)	NM_000969	Hs.180946	90	Protein synthesis, ribosomal protein	Protein biosynthesis	Structural constituent of ribosome; rRNA binding; 5S rRNA binding	Cytosolic large ribosomal subunit; ribosome; intracellular	1.9	0.003
<i>Bos taurus</i> mRNA for similar to ribosomal protein L26	AB098829	Hs.406682	99	Protein synthesis, ribosomal protein	Protein biosynthesis	Structural constituent of ribosome; RNA binding	Cytosolic large ribosomal subunit; intracellular	1.7	0.003
<i>Homo sapiens</i> RALBP1 associated Eps domain containing 1	NM_031922	Hs.333141	94	Signal transduction, endocytosis	—	Calcium ion binding	—	2.1	0.001
<i>Homo sapiens</i> zinc finger protein 36 (KOX18) (ZNF36), mRNA	XM_168302	Hs.24758	80	Transcription regulation	Regulation of transcription, DNA-dependent	Transcription factor activity	Nucleus	2.4	0.004
<i>Homo sapiens</i> transcription factor binding to IGHM enhancer 3	NM_006521	Hs.274184	82	Transcription regulation	Cell growth and/or maintenance; regulation of transcription, DNA-dependent; transcription from Pol II promoter	Transcription factor activity	Nucleus	2.1	0.019
RCO-BN0284-260700-023-09 BN0284 <i>Homo sapiens</i> cDNA <i>Homo sapiens</i> RNP2 RNA-binding region (RNP1, RRM) containing 2	BE818469	Hs.282901	84	Transcription regulation, RNA processing	mRNA splicing; regulation of transcription, DNA-dependent	RNA binding	Nucleus	2.1	0.006
<i>Homo sapiens</i> WD repeat domain 13 <i>Homo sapiens</i> GTF21 repeat domain containing 1	NM_017883 NM_016328	Hs.12142 Hs.430854	88 93	Transcription regulation? Transcription regulation	— Regulation of transcription, DNA-dependent; development	— RNA polymerase II activity, enhancer binding	Nucleus Nucleus	1.9 1.8	0.004 0.009
<i>Mus musculus</i> TAF3 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 140 kDa <i>Homo sapiens</i> , similar to RAB28, member RAS oncogene family <i>Homo sapiens</i> sorting nexin 9	XM_129997 BC018067 NM_016224	XM.129997 Hs.306899 Hs.7905	75 84 81	Transcription regulation Transport, small GTPase, vesicle transport Transport, intracellular, endocytosis	Transcription from Pol II promoter Protein localization	Protein binding; RNA polymerase II transcription factor activity RAB small monomeric GTPase activity SH3/SH2 adaptor protein activity	Transcription factor TFIID complex — —	1.7 1.7 1.6	0.001 0.007 <0.001
<i>Bos taurus</i> Niemann-Pick disease, type C1	NM_174758	Hs.404930	97	Transport, membrane protein, intracellular cholesterol transport, molecular pump?	Intracellular protein transport; cholesterol transport	Sterol transporter activity; intracellular transmembrane receptor activity; hedgehog receptor activity	Lysosome; integral to membrane; membrane fraction	1.6	0.002

Table 2 Continued

Common name	GenBank accession number	UniGene	Homology (%)	Protein function	Gene Ontologies Biological Process	Gene Ontologies Molecular Function	Gene Ontologies Cellular Component	Di:Oe*	P value
Bovine cDNA/gene or homologue									
713065 MARC 6BOV	CB455882	—	100	Unknown	—	—	—	4.4	0.022
<i>Bos taurus</i> cDNA 3'									
<i>Bos taurus</i> ORF1 mRNA	Z24674	—	99	Unknown	—	—	—	3.2	<0.001
IMU602702614.R1	CB168971	Bt.13764	99	Unknown	—	—	—	2.9	0.006
CSEQFXL22									
stomach-omasum <i>Bos taurus</i> cDNA									
534395 MARC 4BOV	BM481842	—	94	Unknown	—	—	—	2.3	0.013
<i>Bos taurus</i> cDNA 5'									
TPA: <i>Homo sapiens</i> chromosome 7	BL00002	—	80	Unknown	—	—	—	2.3	0.008
Length=138409401									
BX093047 Soares	BX093047	Hs.153661	91	Unknown	—	—	—	2.2	<0.001
adult brain									
N2b5HB55Y <i>Homo sapiens</i> cDNA clone									
592942 MARC 6BOV	CB420030	Bt.13766	100	Unknown	—	—	—	2.1	0.005
<i>Bos taurus</i> cDNA 3'									
<i>Homo sapiens</i> PAC clone RP5-1032B10 from 7	AC004455	—	87	Unknown	—	—	—	2.0	0.004
<i>Homo sapiens</i> NMDA receptor-regulated gene 2	NM_024611	HS.145608	84	Unknown	—	—	—	1.9	<0.001
IBE603019914.R1									
CSEQFXN35 kidney	CB166522	Bt.6973	99	Unknown	—	—	—	1.9	0.020
<i>Bos taurus</i> cDNA									
BP250010A10E9	BF046719	—	99	Unknown	—	—	—	1.9	0.002
Soares normalized bovine placenta <i>Bos taurus</i> cDNA									
Human chromosome 16 BAC clone	AC002394	—	77	Unknown	—	—	—	1.8	<0.001
CIT987SK-A-211C6									
<i>Homo sapiens</i> chromosome 5 clone RP11-215G15	AC106760	—	80	Unknown	—	—	—	1.8	0.017
<i>Homo sapiens</i> hypothetical protein LOC283152, mRNA	BC043548	Hs.114777	83	Unknown	—	—	—	1.7	0.004
BP250016A20A10									
Soares normalized bovine placenta <i>Bos taurus</i> cDNA	BF039581	Bt.5872	98	Unknown	—	—	—	1.7	0.002
211208 MARC 2BOV									
<i>Bos taurus</i> cDNA	BE756907	—	99	Unknown	—	—	—	1.5	0.027
Two cDNA fragments without homology to database sequences	—	—	—	Unknown	—	—	—	—	—

*Mean dioestrus:mean oestrus.

Table 3 Results of real-time RT-PCR

Gene	GenBank accession number	Mean ($\pm$ S.E.M.) pg mRNA/ μ g total RNA at oestrus	Mean ($\pm$ S.E.M.) pg mRNA/ μ g total RNA at dioestrus	Oe:Di*	P value	Efficiency of the PCR
TRA1	NM_174700	22.21 $\pm$ 4.24	1.17 $\pm$ 0.31	19.0	0.002	1.99
ERP70	NM_004911	8.37 $\pm$ 1.10	0.51 $\pm$ 0.11	16.5	<0.001	1.86
GRP78/HSPA5	NM_005347	11.64 $\pm$ 1.34	1.32 $\pm$ 0.24	8.8	<0.001	1.93
AGR2	NM_006408	2.09 $\pm$ 0.82	0.23 $\pm$ 0.08	9.1	0.03	1.93
OVGP1	D16639	371.3 $\pm$ 21.3	71.9 $\pm$ 20.1	5.2	<0.001	1.83
C3	NM_000064	0.63 $\pm$ 0.02	2.26 $\pm$ 0.10	0.28	<0.001	1.86
MS4A8B	NM_031457	0.69 $\pm$ 0.07	1.20 $\pm$ 0.06	0.59	0.001	1.95
18S rRNA	—	2250 $\pm$ 140	1950 $\pm$ 140	1.15	n.s.	1.93

*Mean oestrus:mean dioestrus.

Discussion

The objective of this study was to investigate differences in gene expression at the mRNA level in the oviduct epithelium between oestrus and dioestrus to get a first view into the complex regulation of gene expression during the oestrous cycle. For the first time differential gene expression in bovine oviduct epithelial cells was systematically investigated to identify genes that might be involved in the morphological and functional changes of these cells. So far, only few studies have been done to investigate systematically the oviduct epithelium through the oestrous cycle. Yaniz *et al.* (2000) showed that there are changes in the proportions of secretory and ciliated cells during the cycle, especially in the ampulla. As shown previously both mRNA and protein levels of the steroid hormone receptors showed region-specific and cycle-dependent expression differences in the bovine oviduct (Ulbrich *et al.* 2003).

To identify changes in gene expression, subtracted cDNA libraries were prepared for epithelial cells at oestrus and at dioestrus (enrichment of cDNAs of differentially expressed genes). Subsequently, a set of randomly picked clones (1536 of each library) was analysed by cDNA array hybridisation with probes prepared from oviduct epithelial cells of six cyclic heifers, three at oestrus and three at dioestrus, to evaluate reproducibility and biological variation between individual animals. The analysis of the array data showed in general that the average degree of up-regulation of mRNAs at oestrus was more than 2-fold higher than at dioestrus. Sequencing of the cDNAs that showed differential signals revealed 77 different

mRNAs, of which 37 were up-regulated at oestrus and 40 were more abundant at dioestrus.

To verify the results obtained by cDNA array hybridisation seven genes were selected for absolute quantification using real-time RT-PCR. The analysis of the same RNA samples as used for array hybridisation allowed a direct comparison of both methods. The results of the real-time RT-PCR confirmed clearly the data obtained by array hybridisation as shown by the strong correlation of the data. The observation that ratios found by real-time RT-PCR for genes up-regulated at oestrus were higher and the ratios for the selected two genes up-regulated at dioestrus were slightly lower compared with array hybridisation can be easily explained: for the array hybridisation, a ds hybridisation probe was used. Although hybridisation to the cDNA fragment on the array should be preferred, re-association to ds cDNA in the hybridisation solution can occur. The rate of re-association depends on the concentration of each cDNA, which is higher in the probe where the particular cDNA is more abundant. This effect is enhanced if the concentration of the cDNA spotted on the array is not in very high excess to the corresponding cDNA in the hybridisation solution as in the case of highly expressed genes like OVGP1 or TRA1. Furthermore, the mode of background subtraction and normalisation of the raw data could result in normalised values that are slightly too low or too high. This holds as well for the real-time PCR data. In our study the calculation of the ratios was based on absolute mRNA concentrations. Another possibility is to refer the data to a standard like the 18S rRNA. In this case all oestrous values would slightly decrease

and all dioestrous values would increase resulting in slightly lower oestrus:dioestrus ratios. In the present study though this would not have changed the conclusion. In most studies where array hybridisation and real-time RT-PCR results were compared expression ratios were for some of the investigated genes completely different or ratios obtained by array hybridisation were lower (Rajeevan *et al.* 2001, Bangur *et al.* 2002, Jiang *et al.* 2002 (for review see Chuaqui *et al.* 2002)). In contrast, in our study the results for all selected genes correlated very well and the array data were clearly confirmed for the selected genes, demonstrating the capability of array hybridisation on nylon membranes using radioactively labelled probes for the identification of differentially expressed genes.

Only a few of the identified genes have already been described in context with the oviduct or other reproductive organs. The cDNA of the major oviductal glycoprotein (OVGP1) showed by far the highest expression in the oviduct epithelium at oestrus compared with the other identified cDNAs. The measured up-regulation of 2.7-fold is certainly low, even the real-time PCR approach revealed only a 5.2-fold up-regulation. This may be due to the sampling procedure not discriminating between the ampulla and the isthmus region of the oviduct. Recently, we found that the abundance of the OVGP1 mRNA was slightly higher in the contralateral oviduct epithelium compared with the ipsilateral oviduct at day 3.5 (Bauersachs *et al.* 2003). The mRNA of L-plastin, a beta-actin-binding protein (Namba *et al.* 1992), was also found to be regulated by estrogens and progestins in endometrial stromal cells (Leavitt *et al.* 1994). For the 78 kDa glucose-regulated protein (GRP78/HSPA5), a chaperone located in the ER, differential expression in the glandular epithelium of the mouse uterus was described (Simmons & Kennedy 2000). In our study the expression of the GRP78 mRNA was increased 6-fold at oestrus.

Figure 3 Correlation of array hybridisation and real-time RT-PCR data. Seven selected genes identified as up-regulated at oestrus or dioestrus by cDNA array hybridisation were further analysed by real-time RT-PCR using identical RNA samples as for array hybridisation (oestrus $n=3$, dioestrus $n=3$). The mRNA concentrations obtained by real-time RT-PCR (pg mRNA/μg total RNA) and the normalised signals (Hyb signal) were used for the calculation of the correlation coefficient (r). OGP=OVGP1.

Figure 4 Simplified Gene Ontology. Differentially expressed genes at oestrus and dioestrus were classified using the Simplified Gene Ontology function of GeneSpring and functional data obtained by the database analysis. Gene Ontologies for Biological Processes and for Molecular Functions are shown. Bold: up-regulated at oestrus; Italics: up-regulated at dioestrus.

The anterior gradient homologue (AGR2), that was also up-regulated 6-fold, was first identified in *Xenopus laevis* as XAG-2 to play a putative role in ectodermal patterning (Aberger *et al.* 1998). The XAG-2 signalling depends on an intact fibroblast growth factor signal transduction pathway (Aberger *et al.* 1998). Furthermore, this gene was found to be co-expressed with the oestrogen receptor in an oestrogen receptor-positive breast carcinoma cell line (Thompson & Weigel 1998), suggesting that it could be an oestrogen-regulated gene. The abundance of the gastrin-releasing peptide (GRP) mRNA was 4-fold higher at oestrus than at dioestrus. GRP belongs to a group of regulatory peptides and was found to be expressed in the reproductive tract of different species (Happola & Lakomy 1989, Fraser *et al.* 1994, Whitley *et al.* 1996, 1998, Budipitoj *et al.* 2001). In sheep a study of GRP mRNA expression during the oestrous

cycle in the uterus showed that expression was markedly increased at day 16 compared with days 4, 10, 12 and 14 (Whitley *et al.* 1998); day 0 was not investigated. The expression in the uterus was located mainly to the uterine gland epithelial cells (Budipitoj *et al.* 2001).

Also some of the genes up-regulated in the oviduct epithelial cells at dioestrus have been described in the context of reproduction. The expression of the purine nucleoside phosphorylase mRNA that was up-regulated 2.6-fold was also described in human and mouse endometrium (Kao *et al.* 2003). Our previous study of bovine oviduct epithelial cells at day 3.5 of the oestrous cycle revealed a 2-fold higher mRNA level of this gene in the ipsilateral compared with the contralateral oviduct (Bauersachs *et al.* 2003). For the complement component C3 a role in the interaction of human gametes was postulated

(Anderson *et al.* 1993). Furthermore, oestrogen-dependent expression and negative regulation by P₄ of the C3 mRNA was shown in the uterus of rats (Brown *et al.* 1990). However, we found a 4-fold increase in gene expression at dioestrus in the oviduct epithelial cells. The FK506 binding protein 1a, also known as immunophilin FKBP12, has a role in T-cell immunosuppression and it was also shown to increase or initiate the sperm motility in the male reproductive tract (Walensky *et al.* 1998).

For further interpretation and classification of the results, i.e. of the identified differentially expressed genes, a simplified Gene Ontology was drawn. In general, different functional classes were up-regulated at the two analysed times of the oestrous cycle. The up-regulation of genes involved in protein secretion and of four secreted proteins at oestrus correlates with morphological observations, since more secretory cells are present in the ampulla during oestrus compared with dioestrus (Yaniz *et al.* 2000). Moreover, a group of genes mediating differentiation and/or suppression of proliferation (e.g. RARRES1, GRP, AGR2, DMTB1) was found to be up-regulated at oestrus. In contrast, at dioestrus genes promoting proliferation and genes involved in transcriptional regulation were slightly up-regulated. Furthermore, the expression of three immune-related genes was increased at dioestrus, indicating changes in the immune regulation during the oestrous cycle. In conclusion, the Gene Ontology classification revealed different phenotypes of the oviduct epithelium, a more differentiated, secretory phenotype at oestrus and a less differentiated at dioestrus. Since these phenotypes are mainly regulated by the ovarian hormones, the results of this study could also be applied to other epithelia whose development is under the control of these ovarian hormones.

In summary, our study is the first that systematically investigated changes in gene expression patterns at the mRNA level in bovine oviduct epithelial cells during the oestrous cycle and provides a starting point for more detailed analyses. The results of the cDNA array hybridisation have been verified and exactly quantified using real-time RT-PCR. Since the oviduct is organised into different functional compartments a next step would be to compare gene expression in these functional units at different times of the oestrous

cycle or to assign expression of interesting genes to distinct cells of the oviduct epithelium.

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