Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity

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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. This study investigated transcriptome profiles of endometrium samples from day 18 pregnant vs non-pregnant heifers to get insight into the molecular mechanisms involved in conditioning the endometrium for embryo attachment and implantation. Using a combination of subtracted cDNA libraries and cDNA array hybridisation, 109 mRNAs with at least twofold higher abundance in endometrium of pregnant animals and 70 mRNAs with higher levels in the control group were identified. Among the mRNAs with higher abundance in pregnant animals, at least 41 are already described as induced by interferons. In addition, transcript levels of many new candidate genes involved in the regulation of transcription, cell adhesion, modulation of the maternal immune system and endometrial remodelling were found to be increased. The different expression level was confirmed with real-time PCR for nine genes. Localisation of mRNA expression in the endometrium was shown by *in situ* hybridisation for *AGRN*, *LGALS3BP*, *LGALS9*, *USP18*, *PARP12* and *BST2*. A comparison with similar studies in humans, mice, and revealed species-specific and common molecular markers of uterine receptivity.

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Introduction

In cattle, implantation of the embryo takes place after day 18 of gestation and, like in other ruminants, an epitheliochorial placenta is formed through a relatively non-invasive placentation process. In contrast to primates and rodents, in cattle the time of implantation is late, when the trophoblast layer of the conceptus is elongated and fills out the entire ‘pregnant’ uterine horn. Interferon- τ (IFNT) has been identified as the major embryonic pregnancy recognition signal in ruminants, which prevents the induction of luteolysis, thus enabling the establishment of a pregnancy (Bazer *et al.* 1997). This is mediated by the suppression of the expression of oestrogen receptor- α (*ESR1*) and oxytocin receptor (*OXR*) mRNA, which prevents the production and

pulsatile secretion of luteolytic prostaglandin $F_{2\alpha}$ resulting in the maintenance of the ovarian *corpus luteum* and progesterone production (Spencer & Bazer 1996). Maximum secretion of IFNT was observed on day 17 (Bazer *et al.* 1997) in parallel to the time of maternal recognition of pregnancy. IFNT is a member of the type-I IFNs (Martal *et al.* 1998) and is most similar to human IFN- ω (Roberts *et al.* 1999). IFNT mediates its effects in bovine endometrium by binding to endometrial type-I IFN receptors (Li & Roberts 1994), leading to the activation of the JAK–STAT pathway (Binelli *et al.* 2001). A number of genes have already been described as induced by IFNT in the endometrium of cattle or sheep, such as IFN-stimulated gene 15 (*ISG15*), 2'-5'-oligoadenylate synthetase (*OAS*), bovine ubiquitin-activating E1-like enzyme (*UBE1L*), members of the

1–8 family (*IFITM1-3*), MX protein (*MX1*), granulocyte-macrophage colony-stimulating factor-1 (*GM-CSF1*), IFN regulatory factors 1 (*IRF1*) and 2 (*IRF2*) and signal transducer and activator of transcriptions 1 (*STAT1*) and 2 (*STAT2*) (Thatcher *et al.* 2001, Wolf *et al.* 2003). Recently, a database of IFN-stimulated genes revealed by microarray experiments has been established (de Veer *et al.* 2001).

Although the major embryonic pregnancy signal (IFNT) has been known for a long time, the detailed mechanisms of the preparation of the endometrium for the implantation of the conceptus are not completely understood. To investigate these mechanisms in the bovine endometrium, a large project was started using holistic approaches at the transcriptome and the proteome levels (Wolf *et al.* 2003). The present study is a part of this project and uses a combination of subtracted cDNA libraries and cDNA array hybridisation to identify mRNAs of genes differentially regulated in the bovine endometrium by the presence of a conceptus on day 18 of gestation – the preimplantation stage. In mice, rhesus monkey and humans, microarray analyses have been performed to find mRNAs of differentially expressed genes during the embryonic implantation or the window of implantation (Reese *et al.* 2001, Kao *et al.* 2002, Sun *et al.* 2004). These studies revealed numerous genes that are regulated during the preparation of the endometrium for the implantation of the embryo. However, due to the different types (epitheliochorial placentation in cattle vs haemochorial placentation in primates and rodents) and the different times of implantation in cattle and other ruminants compared to these species, a transcriptome study of bovine endometrium at the preimplantation stage is of particular importance. Previously, we have identified genes with higher mRNA levels in the bovine endometrium of day 18 pregnant cows (Klein *et al.* 2006). As biological model, monozygotic twin pairs were used; one twin received two embryos produced *in vitro*, whereas the other received a sham transfer and served as non-pregnant control. In comparison to Klein *et al.*, the present study investigated transcriptome changes in the endometrium, which were induced by an *in vivo* embryo derived from artificial insemination. Furthermore, 140 additional transcripts, which are increased or decreased in pregnant endometrium, were identified considerably expanding the set of genes differentially expressed in bovine endometrium at the preimplantation stage.

Experimental animals

All experiments with animals were conducted with permission from the local veterinary authorities and in accord with accepted standards of Humane Animal Care.

Materials and Methods

Pretreatment of animals and collection of endometrial tissue samples

Ten cyclic heifers (*Bos taurus*, Deutsches Fleckvieh, Simmental) between 17 and 22 months old were synchronised by injecting intramuscularly a single dose of 500 µg Cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at dioestrus. Animals were observed for sexual behaviour (i.e. toleration, sweating and vaginal mucus) to determine standing heat, which occurred around 60 h after Estrumate injection. In addition, the animals were checked with ultrasound-guided follicle monitoring. Blood samples were taken on days 20 and 0 of the oestrous cycle and just before slaughtering to determine serum progesterone (P₄) levels (Prakash *et al.* 1987). The first group of animals was inseminated with cryo-conserved sperm (ejaculate + diluter, 1:10) at oestrus. Animals were slaughtered on day 18 of gestation and intact conceptuses were detected in the ipsilateral uterine horns of the animals used for gene expression analysis (*n* = 4). Control animals (*n* = 6) were inseminated with the supernatant of centrifuged sperm and also slaughtered on day 18 of the oestrous cycle. No conceptus was found in control animals. Sperm was derived from the same bull. The uterus was prepared and opened longitudinally. In pregnant animals, the conceptus was localised and carefully removed. Tissue samples of the caudal part of the ipsilateral uterine horn were used for transcriptome analyses. Samples were carefully cut from the lamina propria of the intercaruncular endometrium with a scalpel and immediately transferred into vials containing 4 ml RNeasy lysis buffer (Qiagen, Crawley, UK) and incubated overnight at 4 °C. Samples were stored at –20 °C until further processing. Tissue samples for *in situ* hybridisation were collected from the same animal.

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 of the ipsilateral caudal part of the uterus was isolated using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and agarose gel electrophoresis respectively. Sixty micrograms of pooled total RNA (corresponding to approximately 1.8 µg mRNA) were used for the preparation of subtracted cDNA libraries for pregnant and control animals respectively. The construction of the libraries was done as previously described (Bauersachs *et al.* 2003, Bauersachs *et al.* 2004) with the modification that no additional driver

cDNA was added at the second step of subtractive hybridisation. For every library, 1536 cDNA clones were randomly picked, the cDNA fragments amplified via PCR and spotted by a spotting robot (Omnigrid Accent; Gene Machines, San Carlos, CA, USA) onto positively charged nylon membranes (Nytran Supercharge; Schleicher&Schuell, Dassel, Germany) (array size, 20 × 50 mm). Array hybridisation with ³³P-labelled cDNA probes was done as previously described (Bauersachs *et al.* 2004). The array hybridisation with ten cDNA probes corresponding to the ten animals was processed simultaneously.

Analysis of array data

Array evaluation was done using AIDA Image Analyzer software (Version 3.52; Raytest, Straubenhardt, Germany). Background was subtracted with the 'Lowest grid dot' function. Raw data obtained by AIDA Array software were exported to Microsoft Excel and normalised to the median of the signals of internal reference cDNAs (60–70 cDNAs that show the lowest coefficient of variation over all hybridisations) for each array. Normalised data were log₂-transformed and analysed with the *significance analysis of microarrays method* (SAM) (Tusher *et al.* 2001). Cut-off was set to twofold and false discovery rate (FDR) to 1%. If a gene was represented on the array by more than one cDNA fragment, the mean signal ratio was calculated. The data discussed in this publication have been deposited in National Center for Biotechnology Information's (NCBI's) Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession number GSE3225.

Sequencing of cDNAs with differential hybridisation signals and data analysis

cDNA fragments set as interesting were sequenced directly from spotting solutions using a pipetting robot (Multiprobe II Automated Liquid Handling System; Perkin Elmer, Rodgau, Germany) and automated DNA sequencing (3100-Avant Genetic Analyzer; Applied Biosystems, Langen, Germany). The resulting sequences were compared with public sequence databases using the basic local alignment search tool ('discontiguous Mega BLAST') at the NCBI (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 bovine genome (http://www.ensembl.org/Bos_taurus/blastview). For weak similarities to human genes, similarity at the protein level was also checked. Based on the human orthologous genes, the most specific gene ontology (GO) categories were determined using the NetAffx Analysis Center (Affymetrix, High Wycombe, UK). The resulting data were

supplemented with additional information from Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) and from the literature, respectively. Enriched GO categories were identified with GO tree machine (GOTM) (Zhang *et al.* 2004) (all human genes as reference gene list) and Bibliosphere (Version 6.03; GenomatiX, Munich, Germany). The z-score revealed by Bibliosphere was calculated as follows: $z = (\text{observed} - \text{expected}) / \text{s.d.}(\text{observed})$. Comparison to other studies was based on human gene IDs for studies in humans and rhesus monkey and on the official gene name for studies in the mouse and in sheep. Gene lists of the corresponding papers were translated with Bibliosphere or other tools to obtain the gene ID and the official gene symbol. The lists were then compared in Microsoft Excel.

Real-time RT-PCR

The same RNA samples as for array hybridisation were used. A two-step quantitative real-time RT-PCR (qPCR) was undertaken as described recently (Klein *et al.* 2006). Quantitative real-time PCRs using the LightCycler DNA Master SYBR Green I protocol (Roche) were performed. The following primers were used to amplify the specific fragments referring to selected regulated genes (length of the PCR products in square brackets): *AGRN* (for 5'-TATGAGTGCCTGTGTCCTGC; rev 5'-CTGCTGGAC-TCGATGTAGGTC [123 bp]); *BST2* (for 5'-ACACTGAACCGTTGTCCTCC; rev 5'-GGGCAGTGGACTCTCTGAAG [145 bp]); *C17orf27* (for 5'-AAGAAGGAAGAAAGC-CAGCTTC; rev 5'-GACTACTGCATGCTGAGAGGG [104 bp]); *DKK1* (for 5'-GCGTCACGCTATGTGCTG; rev 5'-GCTTTCAATAATGGTTTCCTCG [109 bp]); *LGALS9* (for 5'-CTTCAACGAGAATGCGGTG; rev 5'-TTGAAG-CAGTGCCCTTCAC [141 bp]); *STAT1* (for 5'-CTCAT-TAGTTCTGGCACCAGC; rev 5'-CACACGAAGGTGATG AACATG [108 bp]); *DTX4* (for 5'-GTCTCTTCCCGA-GATGCTTG; rev 5'-CATGAGAGAGAGGGCAGAGG [147 bp]); *MTA2* (for 5'-CAGCAGCTCTTCTTGCAAG; rev 5'-AGTGCTTGTGCTCAGCAGTCTC [150 bp]); *OXTR* (for 5'-ACGGTGTCTTCGACTGCTG; rev 5'-GGTGGCA-AGGACGATGAC [110 bp]) and polyubiquitin (Z18245) as a housekeeping gene (for 5'-AGATCCAGGATA-AGGAAGGCAT; rev 5'-GCTCCACCTCCAGGGTGAT [198 bp]).

All the amplified PCR fragments were sequenced to verify the resulting PCR product. Thereafter, the specific melting point (MP) of the amplified product carried out within the LightCycler standard PCR protocol served as a verification of the product identity. The annealing temperature was 60 °C for all the PCRs. The MPs and the appropriate fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment were as follows: polyubiquitin, *AGRN* (MP 88 °C, FA 84 °C), *BST2* (MP 86 °C, FA 75 °C), *C17orf27* (MP 79 °C, FA 75 °C), *DKK1* (MP 83 °C, FA 79 °C), *LGALS9* (MP 89 °C, FA 86 °C), *STAT1* (MP 87 °C, FA

84 °C), *DTX4* (MP 86 °C, FA 83 °C), *MTA2* (MP 90 °C, FA 86 °C) and *OXTR* (MP 87 °C, FA 83 °C). As negative controls, water was used instead of cDNA. The cycle number required for achieving a definite SYBR Green fluorescence signal (=crossing point, CP) was calculated by the second derivative maximum method (LightCycler software version 3.5.28). The CP is correlated inversely with the logarithm of the initial template concentration.

Data analysis of real-time RT-PCR

Results are presented as means $\pm$ S.E.M. ($n=4$ and 6 respectively) (Table 3). The housekeeping gene polyubiquitin showed no statistical difference between pregnant and control animals. Therefore, the CPs determined for the target genes were normalised against polyubiquitin (Δ CP). Differences between pregnant and control animals are expressed by the $\Delta\Delta$ CP as well as the 'mean fold changes qPCR' (Livak & Schmittgen 2001). The normal distribution was tested by the Kolmogorow–Smirnov method, followed by a Student's *t*-test to find significant differences between pregnant and control animals.

In situ hybridisation

In situ hybridisation was performed as previously described (Bauersachs *et al.* 2005). The sequences of the antisense oligonucleotides were as follows: *AGRN*, 5'-TCAAAGGCCAGTGCCTCCAG; *BST2*, 5'-CACACTG AACCGTTGTCCTC; *BUBP43 (USP18)*, 5'-TCCCAGCT AACAGATCACTG; *LGALS3BP*, 5'-ACTGCCCTTCA CAGTGTCAT; *LGALS9*, 5'-AGCAGTGCCCTTCACA CATG and *PARP12*, 5'-AGCACAGGTATTGAGATCTCA. Negative controls were done by omitting the oligonucleotide probe and by hybridisation with sense oligonucleotide probes (complementary sequences of the antisense oligonucleotides).

Results

Identification of differentially expressed genes and validation by qPCR

Total RNA was isolated from endometrial tissue samples derived from the caudal ipsilateral uterine horn of pregnant or non-pregnant heifers respectively. Samples were collected on day 18 of gestation from ten animals (pregnant, $n=4$; control, $n=6$). The determination of serum P_4 levels revealed that the pregnant animals displayed high serum P_4 levels (2.3–5.9 ng/ml) and the control animals had significantly lower serum P_4 levels (0.4–1.6 ng/ml) respectively at the time of slaughtering.

For the enrichment of cDNAs of differentially expressed genes, two subtracted cDNA libraries were produced for pregnant and control animals respectively,

starting from a pool of RNA samples from each group. One thousand five hundred and thirty-six randomly picked cDNA clones of each library were analysed by cDNA array hybridisation with the endometrial tissue samples to detect cDNAs, which reveal different signals between the two experimental groups.

Sequence analysis of 480 cDNA fragments, which resulted from the SAM analysis (fold change ≥ 2.0 , FDR = 1%) revealed 109 different transcripts with higher levels in pregnant animals (in the following referred to as upregulated genes). Nine different major histocompatibility complex (MHC) class-1 mRNAs were counted only as one transcript (Table 1 which can be viewed at <http://www.reproduction-online.org/supplemental/>). In the control group, 70 mRNAs were detected with higher concentrations compared to the pregnant group (in the following referred to as downregulated genes) (Table 2 which can be viewed at <http://www.reproduction-online.org/supplemental/>). The mean fold-up ratio was 6.0 and ranged from 2- (cut-off) to 103-fold in comparison to a mean fold-down of 5.1 that ranged between 2- and 120-fold. Among the upregulated genes, at least 41 have already been described as induced by IFNs. Twenty-four of the downregulated genes were previously detected as upregulated at oestrus compared to the dioestrus stage in bovine endometrium (Bauersachs *et al.* 2005).

Nine genes were selected for mRNA quantification by qPCR (Table 3). The results obtained by array hybridisation were clearly confirmed and more accurate gene expression differences were obtained. Higher expression differences were observed by qPCR in particular for *BST2*, *DKK1*, *LGALS9* and *STAT1*.

GO classification of the genes with higher mRNA levels in pregnant animals

Based on data for the corresponding human genes, the obtained genes were assigned to GO categories to identify relevant functional groups and prominent biological processes. Figure 1 gives a simplified overview for the GO categories based on the major categories 'biological process' and 'molecular function' for the upregulated genes. The subcategories with the highest numbers of genes were: immune response, hydrolase activity, nucleotide binding, signal transduction, transferase activity, transport and peptidase activity. Table 4 (which can be viewed at <http://www.reproduction-online.org/supplemental/>) shows the assignment of genes with higher mRNA levels in pregnant animals to biological processes and molecular functions in more detail for the most interesting categories and considers that many genes can appear in different subcategories providing different (functional) views on a single gene.

GOs that are probably enriched, and therefore, may be particularly interesting, are shown in Fig. 2 for the upregulated genes. Enriched means that there are much

Table 3 Quantification of mRNA levels for selected genes by quantitative real-time RT-PCR (qPCR).

Gene	CP mean/s.e.m.		Δ CP mean/s.e.m.		$\Delta\Delta$ CP	Mean fold change		P-value qPCR	q-value array
	Pregnant	Control	Pregnant	Control		qPCR	Array		
Ubiquitin	17.9 $\pm$ 0.1	17.9 $\pm$ 0.1	—	—	—	—	—	—	—
AGRN	21.7 $\pm$ 0.2	24.1 $\pm$ 0.2	3.6 $\pm$ 0.3	6.3 $\pm$ 0.2	2.7	6.4	4.9	<0.001	0.00
BST2	20.3 $\pm$ 0.6	24.0 $\pm$ 0.5	2.2 $\pm$ 0.5	6.3 $\pm$ 0.5	4.1	16.7	8.3	<0.001	0.00
C17orf27	19.1 $\pm$ 0.3	22.0 $\pm$ 0.3	1.0 $\pm$ 0.4	4.2 $\pm$ 0.4	3.2	9.4	5.4	<0.001	0.00
DKK1	21.9 $\pm$ 0.4	29.2 $\pm$ 1.0	3.8 $\pm$ 0.5	11.4 $\pm$ 1.0	7.6	191.2	11.9	<0.001	0.00
LGALS9	23.0 $\pm$ 0.2	25.7 $\pm$ 0.5	4.9 $\pm$ 0.1	7.9 $\pm$ 0.5	3.0	8.1	3.7	<0.001	0.00
STAT1	19.2 $\pm$ 0.1	22.1 $\pm$ 0.2	1.1 $\pm$ 0.2	4.3 $\pm$ 0.2	3.2	9.2	2.4	<0.001	0.00
DTX4	24.6 $\pm$ 0.0	21.8 $\pm$ 0.2	6.6 $\pm$ 0.1	4.0 $\pm$ 0.2	−2.5	−5.8	−4.4	<0.001	0.00
MT2A	24.6 $\pm$ 0.2	21.8 $\pm$ 0.6	6.5 $\pm$ 0.3	4.0 $\pm$ 0.4	−2.5	−5.7	−6.3	<0.001	0.31
OXTR	28.3 $\pm$ 0.4	21.6 $\pm$ 0.2	10.2 $\pm$ 0.4	3.8 $\pm$ 0.3	−6.4	−84.3	−120	<0.001	0.00

CP, crossing point; Δ CP, CP − CP_{ubiq}; $\Delta\Delta$ CP, Δ CP_{control} − Δ CP_{pregnant}.

more genes observed for a category than expected by chance. For the categories shown in Fig. 2, the z-score provided by Bibliosphere software was at least 2.5 or the P-value calculated by GOTM was less than 0.006 respectively. In addition to the major categories in Fig. 1, the following biological processes or molecular functions were clearly overrepresented: complement activation, regulation of apoptosis, I- κ B kinase/NF- κ B cascade, protein amino acid ADP-ribosylation, endonuclease activity and ATP-dependent helicase activity.

Comparison to related studies

The identified genes were compared to two recent studies of transcriptome changes in the preimplantation phase in cattle (Klein *et al.* 2006) and sheep (Gray *et al.* 2006) (Table 5 which can be viewed at <http://www.reproduction-online.org/supplemental/>). Twelve genes were identified in all three studies and are all known as type-I IFN-induced genes. In the study of monozygotic

twins using cows on day 18 of pregnancy, only genes with higher mRNA levels in pregnant endometrium were analysed. The overlap with this study was 39 genes and the mRNA expression differences were all consistent. Gray *et al.* compared day 14 pregnant, cyclic and uterine gland knockout ewes as well as different treatment groups (progesterone and/or IFNT and/or antiprogesterin and/or control proteins). The overlap with this study was 23 genes. Except for four genes (*ACTB*, *C17orf27*, *GRP* and *HSPA5*), mRNA expression differences were in line compared with the study in the sheep.

Furthermore, the gene lists revealed by the present study were compared to the results of eight related studies in other species, which show an invasive type of embryo implantation. The six studies of human endometrium compared mRNA levels during the putative window of implantation (days 6–10 after luteinizing hormone (LH) surge) with earlier stages of the cycle ranging from 6 days before and 4 days after the LH surge (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.*

Figure 1 Classification of the genes of known function identified as upregulated in the endometrium of day 18 pregnant animals based on the gene ontologies (GOs) *Biological Process* and *Molecular Function*. Multiple naming is possible.

Figure 2 Enriched GO terms for genes upregulated in the endometrium of pregnant animals. Observed vs expected numbers of genes for relevant GO terms are shown.

2003, Riesewijk *et al.* 2003, Mirkin *et al.* 2005). Ace & Okulicz (2004) compared similar stages in the rhesus monkey. Another two studies analysed gene expression profiles during embryo implantation (Reese *et al.* 2001) and oestrogen-induced genes (Ho *et al.* 2004) in mouse endometrium respectively. Fifty-five genes were found overlapping with at least one of these studies (Table 6 which can be viewed at <http://www.reproduction-online.org/supplemental/>). Of these genes, 29 were regulated in the same direction, 17 were contrary and 7 were detected as 'expressed' only.

Localisation of mRNA expression by *in situ* hybridisation

In situ hybridisation with biotinylated oligo-probes was used to detect the mRNAs of six genes that were upregulated in the pregnant animals (Fig. 3). *AGRN*, *LGALS3BP* and *LGALS9* were selected due to their potential role in cell adhesion. *USP18* and *PARP12* stand for protein modification and *BST2* has probably a role in immune modulation. A specific pattern of mRNA distribution was found for each of these genes (Table 7). The hybridisation signal was always confined to cells of the endometrium. No mRNA staining was seen in the myometrium or the serosa. No specific signal was observed in sections hybridised with the sense strand or the section that was incubated with buffer only instead of the oligo-probes. Strong staining for *AGRN*, *LGALS3BP* and *USP18* was found in the luminal epithelium of the uterus, whereas a somewhat lesser intensity was seen for *PARP12* and *LGALS9*. For *BST2*, only a weak content of mRNA was found. The superficial glands generally showed only a weak to moderate reaction for all the genes studied. Only for *PARP12*, a distinct staining was seen both in the superficial and deep uterine glands. The deep glands also showed a pronounced staining for *USP18* mRNA. Most fibroblasts

of the endometrium displayed a distinct hybridisation signal for *PARP12* and a somewhat weaker staining for *BST2* and *USP18*.

Discussion

The characterisation of the molecular mechanisms of attachment and implantation of the bovine conceptus are of particular importance towards the identification of reasons for embryonic mortality in the preimplantation period. To identify genes involved in these processes, mRNA expression profiles of bovine endometrium on day 18 of pregnancy were compared to control samples collected on day 18 of the oestrous cycle. Both groups of animals were prepared under identical conditions to avoid influences of differences in animal treatment on gene expression profiles. To achieve this, the control animals were inseminated with the supernatant of centrifuged sperm of the same bull. However, the heifers used for the collection of endometrial samples showed lower serum P_4 levels in the control group. This is reflected by the upregulation of genes in the control group, which are characteristic for endometrial remodeling during oestrus (see also below). Nevertheless, for the establishment of pregnancy and the implantation of an embryo both are important, the signalling of the embryo and the maintenance of high serum P_4 levels. For this study, the analysis of day 18 was the most important, as it is the time immediately before conceptus implantation. It has to be considered further that the decision for the progression of the cycle (i.e. luteal regression) is already made around day 16 when *OXTR* mRNA starts rising during the normal oestrous cycle.

Differences in mRNA levels were identified using a combination of subtracted cDNA libraries and cDNA array hybridisation. The comparison of four pregnant animals vs six control animals revealed 179 genes with differences in mRNA levels of twofold or more

Figure 3 *In situ* hybridisation with endometrial tissue samples of day 18 pregnant animals. Endometrial sections near the epithelial surface (a), of the deep uterine glands (b) and the corresponding negative (sense) controls (c) are shown. Magnification, 120-fold.

Table 7 Results of *in situ* hybridisation of bovine endometrium.

Gene	Luminal epithelium	Glandular epithelium		Stromal cells
		Superficial	Deeper glands	
<i>AGRN</i>	+++	+	+	+
<i>LGALS3BP</i>	+++	(+)	(+)	–
<i>LGALS9</i>	+ / ++	(+)	(+)	(+)
<i>BUBP43</i> (<i>USP18</i>)	+++	+	++	+
<i>PARP12</i>	++	++	++	++
<i>BST2</i>	+	+	+	+

–, no staining; (+), weak staining; +, moderate staining; ++, distinct staining; +++, strong staining.

(FDR=1%). Nine mRNAs were selected for the verification of the array results and exact quantification by qPCR. Array results were clearly confirmed for these mRNAs. Very high expression differences were underestimated by the array approach for some genes for different reasons (see also Bauersachs *et al.* 2004). Although there was virtually no visible signal of the control probes for *DKK1*, the array evaluation software calculated a signal value because of a higher background at the *DKK1* spot compared to the selected background spot. For *STAT1*, the signals of the control probes were artificially elevated by a very strong signal of the neighbouring cDNA spot resulting in a decreased expression ratio.

At least 41 of the upregulated genes are known as stimulated by IFNs indicating the response of the endometrium to the embryonic IFNT. Several of these genes have already been described in the context of early pregnancy in cattle, like *B2M*, *IFITM1* and *IFITM3*, *ISG15*, *MX*, *OAS1* and *OAS2* and *STAT1* (Thatcher *et al.* 2001, Wolf *et al.* 2003), but for many of these, little information exists regarding the exact function in the context of early pregnancy.

A substantial number of genes (70 genes) with higher mRNA levels in the endometrium of the control animals is related to the physiological processes associated with the progression of the oestrous cycle. Most of these genes are coding for extracellular matrix (ECM) proteins, proteins related to ECM remodelling or proteins of the cytoskeleton reflecting oestrogen-induced changes in the endometrium. In a previous study, 24 of these genes were already detected as upregulated at oestrus compared to the dioestrus stage in bovine endometrium (Bauersachs *et al.* 2005). Furthermore, mRNAs like *ESR1*, *OXTR* and *STC1* are related to the progression of the oestrous cycle in non-pregnant animals and downregulation of *ESR1* and *OXTR* in the endometrium of pregnant animals as a mechanism to prevent the progression of the oestrous cycle has been shown by Spencer & Bazer (1996). Overall, the upregulated genes appeared to be much more interesting regarding biological processes related

to the preparation of the endometrium for the implantation of the embryo. Therefore, the following discussion concentrates mainly on biological processes, where particularly such genes are involved, which had higher mRNA levels in the endometrium of pregnant animals (overview in Fig. 4).

Protein modification processes and NF- κ B cascade

GO classification revealed many genes playing a role in protein modification processes. A couple of genes related to the so-called ISG15ylation process were found. The ubiquitin-like protein, ISG15, is suggested to play an important role in IFNT signalling (Austin *et al.* 2004). ISG15 is conjugated to intracellular proteins and thereby regulates their function. In the present study, mRNAs coding for two known components of the ISG15ylation system, UBE1L, the initiating enzyme (E1) for ISG15ylation (Rempel *et al.* 2005) and BUBP43 (*USP18*), an ISG15-specific protease that cleaves ISG15 from the conjugates, were found as upregulated in the endometrium of pregnant animals. The mRNA expression pattern of *BUBP43* (*USP18*) in the bovine endometrium of day 18 pregnant animals was analysed by *in situ* hybridisation and is similar to that of *ISG15* (Johnson *et al.* 1999) and *UBE1L* (Klein *et al.* 2006). Several mRNAs of putative components of the ISG15ylation system were also identified. The *IFITM1*-like gene and *IFITM3* are coding for proteins containing a conserved E2 motif (Pru *et al.* 2001). C17orf27 and ISGF3G (also described as RNF31) have domains (RING-finger (really interesting new gene) domain or IBR ubiquitin-associated domain respectively) found also in ubiquitin E3 ligases indicating that these two proteins may possess E3 ligase activity. Furthermore, the mRNAs of *DTX4* and *IBRDC2* were decreased in pregnant endometrium. The proteins encoded by these genes also have E3 ligase activity (Takeyama *et al.* 2003, Ng *et al.* 2003). In addition, *BIRC4BP* (*XAF1*) mRNA levels were increased in the endometrium of the pregnant animals. BIRC4BP protein has been described as an antagonist of XIAP (X-linked inhibitor of apoptosis, now named BIRC4; baculoviral IAP repeat-containing 4; Liston *et al.* 2001), an anti-apoptotic protein that possesses E3 ubiquitin ligase activity (MacFarlane *et al.* 2002). The regulation of all these genes, which are potential components of the ISG15ylation pathway, underlines the importance of this process for regulating the response of the endometrium to the embryonic IFNT.

Another protein modification process, ADP-ribosylation, is represented by three members of the poly(ADP-ribose) polymerase (PARP) superfamily (Ame *et al.* 2004). This process is involved, for example, in the regulation of membrane trafficking and actin cytoskeleton (Ame *et al.* 2004). The founding member *PARP1* is involved in DNA repair and synthesises a polymer of ADP-ribose on various nuclear proteins associated with

Figure 4 Schematic picture of processes and corresponding genes probably involved in the preparation of the endometrium for the attachment of the conceptus. STAT1-1, STAT1 homodimers; ISGF3, transcription factor complex consisting of STAT1/STAT2 heterodimer and ISGF3G; genes in solid boxes, upregulated in the endometrium of pregnant animals; genes in dashed boxes, downregulated in the endometrium of pregnant animals.

chromatin in response to DNA damage. Expression of *PARP1* is induced by type-I IFNs. The function of the three members identified in the present study, *PARP9*, *PARP10* and *PARP12*, is not well defined. *PARP9* protein has been implicated in B-cell migration. A link to ubiquitination is found in the ubiquitin interaction motif (UIM) present in *PARP10*. *PARP10* also contains a motif specific for binding to RNAs with the RNA recognition motif found in a variety of RNA-binding proteins. *PARP12* contains a WWE domain found in classes of proteins associated with ubiquitination. *PARP12* has three zinc fingers of the C-x8-C-x5-C-x3-H type, which includes zinc finger proteins from eukaryotes involved in cell cycle or growth phase-related regulation. The UIM domain of *PARP10* and the WWE domain in *PARP12* also suggest a role in the regulation of ISG15ylation in the context of the present study. This is further supported by the *PARP12* mRNA expression pattern in the bovine endometrium of day 18 pregnant animals, which is similar to the pattern of mRNAs coding for ISG15ylation components like *ISG15*, *UBE1L* and *BUBP43*.

Besides these protein modification systems, several genes were identified, which are involved in the regulation of the I-κB kinase/NF-κB cascade. The NF-κB pathway has been shown to play an important role in the regulation of implantation in humans (King *et al.* 2001) and mouse (Nakamura *et al.* 2004). Together with the effects of progesterone, regulation of this pathway

is probably responsible for the control of cytokine production and pro-inflammatory pathways.

Regulation of transcription

The mRNAs of six genes coding for transcription factors were found as upregulated in the endometrium of pregnant animals. *ISGF3G* (*IRF9*) and *STAT1*, coding for two components of the ISGF3 transcription factor complex, play a central role in IFN signalling. ISGF3 binds to IFN-stimulated response elements found in a variety of IFN-induced genes, whereas homodimers of STAT1 bind to the so-called GAS (IFN-γ-activation site) elements (Stewart *et al.* 2001). *IFI16* belongs to the IFN-inducible HIN-200 family (haematopoietic IFN-inducible nuclear antigens with 200 amino acid repeats) and the encoded protein can function as a transcriptional repressor. IFI16 protein is involved in the control of cell cycle regulation via modulation of TP53 (p53) and its target gene regulation (Kwak *et al.* 2003). For NR2F2 (COUP-TFII), a nuclear orphan receptor, active repression of the human oxytocin gene promoter was shown in the uterine epithelial cells (Chu & Zingg 1997). In the mouse, *NR2F2* haploinsufficiency results in decreased progesterone synthesis in the corpus luteum leading to reduced ability of the endometrium to support pregnancy (Takamoto *et al.* 2005). Since the reduced uterine stromal cell function was observed even after

exogenous administration of progesterone, part of the *NR2F2*^{+/-} phenotype is specific to the uterus. A role of *NR2F2* protein in the remodelling of the endometrium through regulation of normal vascularisation has been suggested. The haploinsufficiency model of the *NR2F2* gene indicates that the encoded transcription factor is essential for normal female reproduction, in particular, for endometrial functions during the peri-implantation period. Furthermore, *EPAS1* mRNA was found as upregulated in the endometrium of pregnant animals. *EPAS1* codes for a basic-helix-loop-helix/PAS domain transcription factor, which has been shown to promote angiogenesis through transactivation of vascular endothelial growth factor, its receptors and several other genes (Takeda *et al.* 2004).

Cell adhesion

GO classification assigned six genes, which were found as upregulated in the bovine endometrium of pregnant animals to the process of cell adhesion: *AGRN*, *CD81*, *CLDN4*, *LGALS3BP*, *LGALS9* and *TGM2*. Agrin is a heparan sulphate proteoglycan and involved in the formation of synapses of the neuromuscular junction and the immunological synapse (Bezakova & Ruegg 2003). Agrin might have a general role in the formation of very close cell contacts and subsequent signalling between the attached cells. *In situ* hybridisation showed strong staining for *AGRN* mRNA in the luminal epithelium in pregnant animals. *CD81* is a member of the transmembrane 4 superfamily (tetraspanin family) and the encoded protein is involved in cell adhesion as well as in a variety of different biological processes (Levy *et al.* 1998). *CLDN4*, coding for a tight junction protein, is more likely to be responsible for the cell adhesion between epithelial cells of the endometrium regulating, for example, permeability of the epithelium (Tsukita & Furuse 2002) (see below). The IFN-stimulated gene, *LGALS3BP*, codes for a cell-adhesive protein of the ECM, which self-assembles into ring-like structures and binds $\beta 1$ integrins, collagens and fibronectin (Sasaki *et al.* 1998). A possible role of *LGALS3BP* protein for adhesion of the conceptus is further supported by the *in situ* hybridisation results that show nearly exclusive expression in the luminal epithelium. *LGALS9* (galectin 9) is also stimulated by IFNs and the encoded protein was shown to mediate cell aggregation and cell adhesion (Hirashima *et al.* 2004). The mRNA expression pattern in the endometrium revealed by *in situ* hybridisation is similar to that of *LGALS3BP*. A further galectin, galectin-15 was identified in ovine endometrial epithelium and hypothesised to regulate trophoblast migration, adhesion and differentiation (Gray *et al.* 2004). Possibly, galectin-9 has a similar role in bovine endometrium. *TGM2* codes not only for a multifunctional protein that regulates cell adhesion via mediation of the interaction of fibronectin and integrins,

but is also involved in the ECM remodelling, signal transduction and regulation of apoptosis (Fesus & Piacentini 2002). Furthermore, *IFITM1* might function in the adhesion of the conceptus, since *IFITM1* protein was shown to regulate homotypic adhesion in B-lymphocytes (Evans *et al.* 1993).

Remodelling of the endometrium preparing for implantation of the embryo

As mentioned earlier, the transcription factors *NR2F2* and *EPAS1* may be involved in the process of endometrial remodelling during implantation of the embryo. Furthermore, elevated levels of *CLDN4* mRNA were found in the endometrium of pregnant animals in the present study. Claudins are major cell adhesion molecules in tight junctions involved in intercellular sealing in simple and stratified epithelia (Tsukita & Furuse 2002). Claudin 4 was found to selectively decrease Na⁺ permeability in tight junctions. Furthermore, in four microarray studies of human endometrium, *CLDN4* mRNA was found elevated at the window-of-implantation time (Carson *et al.* 2002, Kao *et al.* 2002, Borthwick *et al.* 2003, Riesewijk *et al.* 2003) (see below). In contrast, the mRNA level of *CLDN10* is decreased in the endometrium of pregnant heifers and recently, we showed that *CLDN10* mRNA is strongly upregulated in the bovine endometrium on day 0 of the oestrous cycle (Bauersachs *et al.* 2005). These findings suggest an important role for claudins in the preparation of the endometrium for implantation through the regulation of the architecture of the uterine epithelium. *MEP1B* is coding for a metalloprotease of the astacin family, which was shown to degrade components of the ECM (Kaushal *et al.* 1994). *TGM2* was shown to be involved in wound healing and angiogenesis. Assembly and remodelling of the ECM in different tissues are mediated by cross linking various ECM proteins such as fibronectin, proteoglycans, collagen V, osteonectin, laminin, nidogen and osteopontin and the covalent modification and activation of several growth factors. This important role of *TGM2* in remodelling of the endometrium is further supported by the finding that *TGM2* mRNA is upregulated in bovine endometrium on day 12 (dioestrus) compared to day 0 (oestrus) of the oestrous cycle (Bauersachs *et al.* 2005). Among the IFN-induced proteins, *MX1* was suggested to function as a regulator of endometrial secretion and uterine remodelling (Hicks *et al.* 2003). In the ovine uterus, the expression of the mRNA for hepatocyte growth factor (*HGF*) was detected in the stromal cells and the mRNA of its receptor (*MET*) exclusively in luminal and glandular epithelial cells (Chen *et al.* 2000). Hepatocyte growth factor has been suggested to stimulate epithelial morphogenesis in preparation for the establishment and maintenance of pregnancy, conceptus implantation and placentation. In the present study, *MET* mRNA level was elevated in the endometrium of pregnant animals. Legumain (LGMN), a asparaginyl-specific cysteine proteinase, has been shown to activate

progelatinase A (MMP2) *in vitro* and in cultured cells indicating a role in the regulation of ECM remodelling (Chen *et al.* 2001). Furthermore, mRNA of *TIMP1*, coding for a tissue inhibitor of metalloproteinases, was decreased in the endometrium of pregnant animals compared to the control group. Salamonsen *et al.* suggested an important contribution of MMPs and TIMPs to the marked endometrial remodelling associated with early placentation (Salamonsen *et al.* 1995).

Modulation of the maternal immune system

A stringent regulation of the maternal immune system is very important to protect the conceptus, which can be seen as a semi-allograft. Several genes were identified, which are probably involved in this process. The multifunctional protein galectin-9 was shown to induce apoptosis of various immune cells, including activated CD4⁺ and CD8⁺T-cells, through the Ca²⁺-calpain-caspase-1 pathway, indicating a role in immune modulation by inducing apoptosis of these cells (Hirashima *et al.* 2004). However, in contrast to galectin-9, LGALS3BP has been shown to stimulate natural killer (NK) cell and lymphokine-activated killer cell activity (Ullrich *et al.* 1994). CD81 is reported to influence activation, proliferation and differentiation of B, T and other cells. Furthermore, association with IFITM1 (Leu-13, 9–27) protein was shown on B-cells (Levy *et al.* 1998). IFITM1 itself was shown to suppress the activity of NK cells (Yang *et al.* 2005), suggesting a role in preventing maternal rejection of the fetal semi-allograft. *BST2* was suggested to be involved in pre-B-cell growth (Ishikawa *et al.* 1995). The *in situ* hybridisation results revealed strongest expression in stromal cells. The upregulation of the mRNAs coding for the complement components C1R and C4A and the C1 inhibitor SERPING1 (Ratnoff *et al.* 1969) reflects the regulation of the complement cascade. Co-localisation of *C1R*, *C1S* and *SERPING1* mRNA in the endometrium of day 18 pregnant cows was previously shown (Klein *et al.* 2006).

Comparison to similar studies in cattle and sheep and related studies in humans, mice and primates

The comparison of the known genes with our recent study of monozygotic twin cows (Klein *et al.* 2006) revealed a substantial and consistent overlap confirming the reliability of the results. For this comparison, it has to be considered that in Klein *et al.*, a different biological model was used. Moreover, in addition to SSH, a second subtraction technique was applied, and different statistical analyses were conducted in Klein *et al.* In the study of ovine endometrium (Gray *et al.* 2006), the effects of IFNT and progesterone on gene expression were analysed in addition to the investigation of day 14 pregnant vs

control ewes. Overall, 23 genes were found to overlap between the ovine and the present study. The expression of genes with higher mRNA levels in the bovine endometrium of pregnant animals was mostly detected as IFNT-dependent. The consistency of differential gene expression between cattle and sheep reflects similar mechanisms of pregnancy recognition and similar type of placentation of these two species.

Despite the distinct differences in the biology of reproduction between mammalian species, some genes could be identified, which were regulated similarly in the bovine endometrium compared to the endometrium of humans and rhesus monkey during the putative window of implantation or the mouse at the implantation stage respectively. Therefore, some common regulatory processes in these species can be suggested. For example, *DKK1*, coding for an inhibitor of WNT signalling (Glinka *et al.* 1998), has been found as induced in four human studies at the window of implantation time. In bovine endometrium, *DKK1* mRNA was 12-fold upregulated in the endometrium of pregnant animals. Likewise, for *CLDN4*, higher mRNA levels were detected in human endometrial tissue samples in four studies and in bovine endometrium. Elevated mRNA levels were also found in two human studies for *P8*, *SLC1A1* and *C10orf10* (*DEPP*), coding for a protein that is suggested to modulate the effects of progesterone during decidualisation (Watanabe *et al.* 2005). There may also be some common regulations of the maternal immune system indicated by the consistent regulation of *C1R*, *SERPING1* and *TAP1*. Furthermore, *TGM2*, coding for a protein involved in cell adhesion and ECM remodelling (see above), was shown to be upregulated in the human endometrium during the window of implantation. Comparable regulation in other species was found for a number of downregulated genes as well.

In conclusion, this comprehensive study of transcriptome changes in bovine endometrium at the preimplantation stage revealed a set of very promising candidate genes involved in crucial biological processes like cell adhesion, endometrial remodelling, regulation of the maternal immune system and the response to IFNT, the embryonic pregnancy recognition signal. Furthermore, with the set of genes identified in the present and our previous studies (Bauersachs *et al.* 2005, Klein *et al.* 2006) pregnancy signalling in bovine endometrium can also be compared between normal and pathological states or *in vivo* derived and *in vitro* produced embryos.

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