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Department of Veterinary Sciences
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Dynamic transcriptome profiling of bovine endometrium during the oestrous cycle

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Dynamische Transkriptionsprofile während des Sexualzyklus im Endometrium beim Rind

Inaugural-Dissertation
zur Erlangung der tiermedizinischen Doktorwürde
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1 Introduction

Fertility problems have been among the main reasons for the slaughter of dairy cows for many years. In Germany and Austria infertility is also an important reason for culling of high-performance dairy cows. Current statistics estimate the percentage of female cattle slaughtered due to fertility problems or infertility between 22 and 25% (Arbeitsgemeinschaft Deutscher Rinderzüchter, 2006). Reduced fertility of a herd results in additional costs for artificial insemination and veterinary treatment. An elongated empty period leads to extended lactations, often with reduced milk yield. In addition there are problems due to the lack of replacement heifers. Since these problems result in financial losses for the farmer and retard genetic progress, genetic improvement of fertility would be of great benefit (Gredler 2006). However, improvement of fertility via genetic selection is hampered by the low heritability (between 0.01 and 0.05) of currently recorded fertility traits, e.g. the non-return rate 90 days after artificial insemination. Besides the non-return rate a series of physiological supporting markers, which were partly shown to be marginally related with fertility, are discussed for the selection of fertile cows. These surrogate markers include the progesterone levels in the milk, the locomotor activity and the body temperature, which could enhance the efficiency of the recognition of standing heat. Also measurement of diverse components of the milk as well as a *body condition score*, which have been recommended as additional markers for the evaluation of fertility breeding values. However, these parameters are only indirectly related to the physiology and pathology of reproduction. Thus the search for new markers, which are directly associated with bovine fertility, is necessary. One idea to find such markers is to identify gene expression patterns in the endometrium that correlate with maternal fertility (Wolf, Arnold *et al.* 2006).

The aim of this work was to identify genes with significant changes of mRNA concentrations in the bovine endometrium during the oestrous cycle as an important basis for future studies of endometrial transcriptome changes in normal reproductive physiology vs. pathologic conditions.

2 Approaches for the analysis of mRNA expression levels

Mammalian genomes contain approximately 23,000 protein-coding genes and almost every cell contains this all-embracing genomic information. But every single cell type and also different tissues or physiological stages show a typical pattern of active genes, which are transcribed. Thus, valuable information about the regulation and the nature of biological processes at the molecular level can be deduced from analyses of differences in expression levels of mRNAs between different physiological states, such as stages of the oestrous cycle.

For the analysis of small sets of candidate genes techniques like quantitative RT-PCR, *in situ* hybridisation and Northern blotting are used. However, for holistic analyses highly parallel techniques are applied which are either hybridisation- or sequencing-based. The first high-performance sequencing platform was Massive Parallel Signature Sequencing (MPSS) (Reinartz, Bruyns *et al.* 2002). A sensitivity of a few mRNA molecules per cell was achieved by counting the frequencies of millions of short mRNA-derived sequence tags. Most recently, important advances have been made in speeding up DNA sequencing with the development of the so-called “next generation” sequencing instruments (Roche/ 454: Genome Sequencer FLX (Margulies, Egholm *et al.* 2005); ABI: SOLiD system; Illumina: 1G Genome Analyzer) that are able to produce millions of sequence tags per run. A short survey of current developments of next generation sequencing systems is presented in (Shaffer 2007). Due to their high performance, these new technologies are particularly suited for the analysis of mammalian transcriptomes and will enable the detection of rare transcripts in complex tissues like the endometrium in the future.

The currently most widespread method for the analysis of mRNA expression levels is the hybridisation of microarrays. The microarray technology is based on the specific hybridisation of two complementary DNA strands or of complementary DNA and RNA sequences. Nucleic acids specific for certain mRNA sequences are immobilised as probes on a solid support and hybridised with labelled nucleic acids derived from mRNA isolated from the cells or tissues of interest. The amount of bound label corresponds to the concentration of the corresponding transcripts in the biological sample. There are several commercial microarray platforms performing genome-wide analyses by measurement of the transcription levels of several ten thousands of genes in a single experiment.

Despite the high performance of microarray-based approaches, the reliability of measurements was often challenged due to inconsistent results that have been reported with different microarray platforms. However, the MicroArray Quality Control (MAQC) project has recently investigated a variety of microarray platforms and alternative technology platforms (Shi, Reid *et al.* 2006). Reproducible measurement of gene expression at multiple test sites was achieved as well as a high concordance with different platforms. Detailed analyses were done to compare performance of one- and two-colour systems (Patterson, Lobenhofer *et al.*

2006). Furthermore, the performance of five commercial microarray platforms and the impact of different normalisation methods for expression data were examined (Shippy, Fulmer-Smentek *et al.* 2006). In order to determine the reliability of the quantification of gene expression, DNA microarray results were evaluated with three different quantitative gene expression platforms (Canales, Luo *et al.* 2006). High correlation was observed between quantitative gene expression values and microarray platform results. The only shortcoming of the different microarray platforms was the limited and variable sensitivity for detection of rare transcripts, which interferes with reproducible measurement of differentially expressed genes. Nevertheless, optimisation and standardisation of methods have enhanced the reproducibility of the results across platforms.

The Affymetrix GeneChip® system is a widely used commercial platform which provides a number of arrays for domestic animals, such as cattle, pig, dog and chicken. The design of the Bovine Genome Array was based on content from Bovine UniGene Build 57 (March 24, 2004) and GenBank® mRNAs. The GeneChip® Bovine Genome Array was developed through the GeneChip® Consortia Program, and contains 24,027 probe sets. Based on the original annotation (March 2004) these probe sets represented over 23,000 bovine transcripts and included approximately 19,000 UniGene clusters. However, according to the current state of the bovine Genome annotation and the current content of GenBank® the Bovine Genome Array represents only approximately 15,000 bovine genes (personal communication – Volker Hintermair, Department of Practical Informatics and Bioinformatics, LMU Munich). Gene specific oligonucleotides (from the 3' untranslated region of the mRNA) are synthesised directly on quartz wafers by a photolithographic process. Hybridisation of the arrays is carried out with biotinylated cRNA, which can be detected after hybridisation through fluorescently labelled streptavidin (www.affymetrix.com).

Large microarrays such as the Affymetrix Bovine Genome Array are well suited for the screening for differentially expressed genes in a broad range of applications. For more specialised applications like the analysis of dynamic mRNA expression profiles in bovine endometrium during the oestrous cycle or early pregnancy smaller and more flexible arrays mainly containing probes for the genes of interest (i.e. the differentially expressed genes) would be beneficial. Furthermore, the application of a custom-made array based on spotted cDNAs or oligonucleotides for the analysis of gene expression of a limited number of genes would be less expensive and would simplify the interpretation of expression data. Based on a series of differential gene expression studies of bovine endometrium (different stages of the oestrous cycle, d 18 pregnant vs. nonpregnant) and oviduct epithelial cells (different stages of the oestrous cycle), a custom-made Bovine Oviduct and Endometrium (BOE) cDNA array was developed as a basis for the analysis of endometrial mRNA expression profiles corresponding to normal reproductive physiology and in pathological conditions (Publication 1).

3 The bovine uterus

The function of the bovine uterus is to supply the conceptus - via the contact with the fetal membranes - with oxygen and nutrients. At the end of the pregnancy the uterus has to drive out the calf during parturition. All these processes occur under complex hormonal regulation. Due to its morphology, the bovine uterus is referred to as uterus bicornis. It consists of two uterine horns (cornua uteri), the uterine body (corpus uteri), and the uterine neck (cervix uteri). These horns are two tubes with cranial decreasing diameter, which are lying in parallel in the first part next to the uterine body. Both horns are rolled-up like a snail. The uterine walls consist of the endometrium with the luminal epithelium (LE) in one or more layers and the propria with the uterine glands and connective tissue, beneath the endometrium the tunica muscularis or myometrium and the tunica serosa or perimetrium. The endometrium is subdivided in caruncular and intercaruncular parts. The main difference between these parts is the lack of uterine glands in the caruncles. After implantation of an embryo, the 75 to 120 caruncles grow rapidly and form the placentomes, tight connections between the endometrium and the fetal parts of the placenta (cotyledons) facilitating the exchange of small molecules (Nickel, Schummer et al. 1999).

Implantation in cattle is non-invasive (placenta epitheliochorialis) and follows an extended pre-implantation period where most embryo deaths occur (Burghardt, Johnson et al. 2002). Only a limited number of trophoblast cells show a fusion with luminal epithelial cells forming so-called trophoblast giant cells. There are dramatic differences compared to the early and invasive implantation in rodents and humans. During the oestrous cycle there is also no decidualisation, which is present in primates. Due to the extended pre-implantation period the endometrium secretes histotroph, an uterine secretion product containing e.g. uteroferrin and protease inhibitors to nourish the conceptus for proper development (Roberts and Bazer 1988). During the pre-attachment period in ruminant species the hatched blastocyst elongates rapidly to form a filamentous conceptus that signals for maternal recognition of pregnancy (Ruesse and Sinowatz 1998). The biochemical events of the pre-implantation period provide a unique time span, absolutely critical for embryonic survival, to study the adhesion and signal transduction events associated with apposition and attachment of the conceptus to the endometrium.

3.1 Hormonal regulation of the bovine oestrous cycle

Throughout the oestrous cycle the endometrium features numerous functional and morphological changes. The length of the cycle is normally 21 days and is controlled by the ovarian sex steroid hormones, oestrogen and progesterone. Other important factors in regulation of the oestrous cycle are prostaglandins mainly the luteolysin prostaglandin F2

alpha (PGF2α), which is produced in the endometrial luminal and superficial glandular epithelium (GE) (Spencer, Johnson et al.).

At oestrus (day 0), oestrogen levels peak from the oestrogen-producing dominant follicle resulting in increased ribosomal RNA and DNA synthesis and cell proliferation (Jackson and Chalkley 1974) via an increase in uterine estrogen receptor alpha (ESR1), progesterone receptor (PGR), and oxytocin receptor (OXTR) expression (Spencer, Johnson et al.). Furthermore, oestradiol increases blood circulation and oedematisation of the mucosa, and the smooth musculature shows high contractility. The highly cuboidal endometrial epithelium consists of both ciliated and secretory cells, the latter producing oestral mucus. The uterine glands are elongated as a result of the mucosal oedematisation (Ruesse and Sinowatz 1998). In addition, oestradiol causes a rapid and high increase of luteinising hormone (LH) (Kesner, Padmanabhan et al. 1982), which in turn is necessary for ovulation and formation of the ovarian corpus luteum (CL) (Duffy, Crowe et al. 2000).

During the three days period of metoestrus, oestradiol levels decrease continuously. Following ovulation progesterone levels slowly increase and the progesterone from the newly formed CL stimulates accumulation of phospholipids in the luminal and superficial glandular epithelium that can liberate arachidonic acid for synthesis and secretion of PGF2α (Spencer, Johnson et al.). This phase is additionally characterised by low levels of both progesterone and oestradiol in the peripheral blood. Mucosal oedema and contractility of the smooth musculature are declining during metoestrus.

In dioestrus the surface epithelial cells are flat and the highly active and proliferating uterine glands secrete uterine milk, also called histotroph (Ruesse and Sinowatz 1998). Progesterone levels are highest and act via PGR to block expression of ESR1 and OXTR in the endometrium (Spencer, Johnson et al.). The mature CL produces oxytocin, too. During late dioestrus (from day 15 of the oestrous cycle) progesterone causes the downregulation of its own receptor in the LE and the GE. In consequence, expression of OXTR and ESR1 rises. Binding of oxytocin to its receptor initiates the pulsatile secretion of PGF2α. PGF2α is produced from membrane phospholipids by phospholipase A2, cyclooxygenase, and prostaglandin F2α synthase and is in addition to the regulation of other processes, such as ovulation, implantation and parturition, responsible for luteolysis (Goff 2004; Silvia, Lewis et al. 1991). The functional and structural regression of the CL leads to the decrease of P4 levels. Growing follicles on the ovary release estrogens, resulting in the production of pituitary follicle-stimulating hormone (Hewitt, Deroo et al.) (Kesner, Padmanabhan et al. 1982). FSH stimulates one follicle to growth over a certain extent and to become the dominant follicle for the next ovulation, which produces oestradiol and is responsible for the LH surge before ovulation.

3.1.1 Progesterone

In all mammals progesterone plays an important role in the regulation of the oestrous cycle and acts via the progesterone receptor (PGR), which is expressed in the endometrial epithelium and stroma during the early luteal phase, allowing direct regulation of a number of genes. Progesterone has been shown to be a very important factor for the establishment and maintenance of pregnancy, due to its critical role for conceptus survival and development by orchestrating the biochemical interactions between the embryo and the endometrium. Implantation in cattle starts at the end of dioestrus from day 18 or 19 on. The progesterone-dominated pre-implantation period from blastocyst formation until successful maternal recognition of pregnancy is a very interesting and sensible phase, because up to 40% of total embryonic losses in cattle occur between days 8 and 17 of pregnancy. This early embryonic mortality is the main source of reproductive wastage (Humblot 2001) and results from failures during the development of the early embryo or insufficient supply of the embryo by the endometrium. In this phase the hatching and elongation of the blastocyst proceeds as it undergoes dramatic changes in size and morphology (Spencer and Bazer 2002).

High concentrations of P4 from days 2 to 5 enhance conceptus development and size on day 14 in heifers (Garrett, Geisert et al. 1988), while animals with low concentrations in the early luteal phase have retarded embryonic development in ruminants (Mann and Lamming 2001; Nephew, McClure et al. 1991) and decreased production of interferon tau (IFNT), the signal for establishment of pregnancy, from bovine conceptuses (Mann and Lamming 2001). Both in lactating dairy cows and in heifers, there is a strong positive correlation between early luteal phase plasma progesterone concentrations and embryonic survival rate (Larson, Butler et al. 1997; Starbuck, Darwash et al. 1997; Villa-Godoy, Hughes et al. 1988).

The direct action of progesterone on the uterus is important for the regulation of a number of physiological systems: the endometrial luteolytic signal via prostaglandin F2α ($PGF_{2\alpha}$) synthesis; maternal recognition of pregnancy whereby the conceptus produces IFNT that acts on the endometrium to prevent the luteolytic cascade; and the expression of the appropriate genes responsible for the uterine production of specific proteins, growth factors and modulators of immune functions necessary for conceptus growth and development (Spencer, Johnson et al. 2004).

A number of genes in the rodent, human, and primate uterus are already known to be directly regulated by progesterone, including transcription factors (runt-related transcription factor 2), growth factors (epidermal growth factor), binding proteins (insulin-like growth factor binding proteins), homeobox genes (HOXA10 and HOXA11), morphogens (Indian hedgehog homolog), enzymes (histidine decarboxylase), protease inhibitors (serine peptidase inhibitor, Kazal type 3), peptide hormones (proenkephalin; calcitonin), biogenic amines (histamine), and adhesion proteins (immunoresponsive gene 1) (Spencer, Johnson et al.).

One example for an important pathway regulated by progesterone is the Wnt signalling pathway. In human endometrium dickkopf 1 (DKK1) and other inhibitors of Wnt signalling are markedly upregulated during the progesterone dominated 'window of implantation'. In contrast, secreted frizzled-related protein 4 (SFRP4), another inhibitor, is down-regulated. Presumably the Wnt family plays a role in epithelial-embryo and/or epithelial-stromal interactions and thus in uterine receptivity (Kao, Tulac et al. 2002).

3.1.2 Oestradiol

The steroid hormone 17β-oestradiol is involved in the development of the reproductive tract and the mammary gland, regulates the oestrous cycle, and controls lactation (Watanabe, Suzuki et al. 2003). The effect of oestradiol on the uterus is dependent on P4, because it is only observed after the endometrium has been primed with P4 for a certain period of time (Goff 2004). Retinoids, especially all-trans retinoic acid (RA), suppress oestrogen-dependent proliferation of mammary gland and endometrial cells (Deng, Shipley et al. 2003). The number of oestrogen receptors (ESR1) increases at the end of the luteal phase in the bovine endometrium, due to the loss of PGR expression in the LE and GE and induction by oestradiol (Goff 2004). This increase causes several physiological and biochemical responses, which have been divided into events that occur early, within the first hours after oestradiol elevation, and subsequent responses that follow up to 24 h after the oestradiol peak. Early events are e.g. hyperaemia and infiltration of immune cells such as macrophages and eosinophiles. Genes involved in these processes are for example genes which are known to be induced by growth factors like early growth response 1 (EGR1) or polo-like kinase 2 (PLK2) (Watanabe, Suzuki et al. 2003). Later phase responses include an increase in uterine wet weight, the development of the epithelial layer into columnar secretory epithelial cells, and subsequent mitosis. Further known oestradiol-regulated genes found in a study in mice are involved in chromatin structure or modification (histone cluster 2, H2ac (HIST2H2AC), histone deacetylase 5 (HDAC5)), in keratinisation or cornification (small proline-rich protein 1A and 2A, stratifin) or apoptosis associated (BCL2-antagonist of cell death, v-akt murine thymoma viral oncogene homolog 1) (Hewitt, Deroo et al. 2003).

An important pathway regulated by oestradiol is the transforming growth factor beta (TGFB) pathway. In mammals, three isoforms of TFGB (TGFB1, 2, and 3) have been identified. TGFBs are multifunctional cytokines that influence numerous cellular processes. They can regulate cell proliferation and differentiation positively or negatively depending on cell type and have been implicated in diverse physiological events such as angiogenesis, immune function, steroidogenesis, and tissue remodelling and repair. The regulation of TGFB differs between species. In women, TGFB expression is upregulated during times of increasing plasma progesterone concentrations whereas in rodents and ewes oestradiol increases the endometrial expression of mRNAs encoding TGFB. The high expression rate in late

dioestrus and pro-oestrus reported in rats and ewes may indicate a role for these growth factors in the restructuring of the endometrium that occurs during the transition from one cycle to the next. The TGFBs are recognized to regulate cellular processes through their stimulatory action on extracellular matrix (ECM) formation, cellular matrix binding receptors and increased synthesis of protease inhibitors and repression of proteases that degrade ECM. During pregnancy uterine TGFBs are involved in processes like apoptosis, trophoblast attachment, growth, differentiation, immunotolerance, cytokine and hormone production and embryogenesis (Godkin and Dore 1998).

3.2 Global gene expression studies during the female sexual cycle

The endometrium is a uniquely dynamic tissue, with the capacity to undergo dramatic remodelling in response to cyclic variations in steroid hormones and local autocrine and paracrine factors. The mRNA content of each of its different cell types is altered during different phases of the sexual cycle, with the onset of implantation, as well as in pathological conditions. In human e.g. abnormal menstruation, infections or cancer are known to cause pathological changes in gene expression. Gene expression profiling has the capacity to identify new targets for the manipulation of fertility and the diagnosis and treatment of endometrial abnormalities (White and Salamonsen 2005).

Several microarray studies have been performed to obtain detailed knowledge about the gene expression changes during the physiological female sexual cycle. In humans, several studies were done investigating gene expression changes occurring in the endometrium during the menstrual cycle. Two studies using commercially available microarrays compared gene expression differences between the early secretory phase (2-4 days after LH-surge) and the mid-luteal phase (days 7-9 after LH-surge) (Borthwick, Charnock-Jones et al. 2003; Martin, Dominguez et al. 2002; Riesewijk, Martin et al. 2003). Riesewijk et al. identified 211 regulated genes, 153 were up-regulated at LH+7 versus LH+2, whereas 58 were down-regulated. In addition to genes with already known roles in human endometrial receptivity, the authors found among others genes coding for enzymes, cell cycle-associated proteins and membrane proteins (Borthwick, Charnock-Jones et al. 2003; Martin, Dominguez et al. 2002; Riesewijk, Martin et al. 2003). A study by Borthwick and co-workers identified 149 transcripts with significant differences between the proliferative and the secretory phase of the human menstrual cycle. Among the differential expressed transcripts more than 100 known genes could be subdivided into functional families like transcription factors, cell death and survival factors, transport and carrier proteins and differentiation and embryonic polarity mediators such as members of the Wnt family. Among the remaining transcripts, which were not yet described as related to endometrial functions a number of novel factors were discovered. Examples for these new factors are trefoil factor 3 (TFF3) and glutathione peroxidise 3 (GPX3) (Borthwick, Charnock-Jones et al. 2003; Martin, Dominguez et al. 2002;

Riesewijk, Martin et al. 2003). Endometrial gene expression during the proliferative phase versus the secretory phase of the sexual cycle was also compared in the rhesus monkey (Ace and Okulicz 2004) and in the mouse (Tan, Li et al. 2003). In the endometrium of rhesus monkeys 108 genes were found as significantly regulated. Of these genes 39 were up-regulated at days 21-23 versus day 13, and 69 were down-regulated. The up-regulated genes encode immune-related proteins, cytokines, histones, cell-cycle-regulation proteins, and cell growth inhibitors. The down-regulated genes belong to different functional groups including cell adhesion, extracellular matrix components and inhibitors of Wnt signalling (Ace and Okulicz 2004). Tan et al. compared gene expression profiles of murine endometrium at oestrus versus dioestrus stage. They identified 51 up-regulated and 51 down-regulated genes at oestrus. Most of the up-regulated genes encode secreted immune-related proteins, proteinases and their inhibitors, indicating their potential involvement in sperm viability as well as capacitation. The down-regulated genes mainly encode cell cycle-related factors, reflecting the active proliferation of the endometrium at dioestrus (Tan, Li et al. 2003). Peter Rogers group was the first that collected biopsy samples of human endometrium to specify stages of the cycle based on mRNA expression profiles (Ponnampalam, Weston et al. 2004). Talbi et al. (Talbi, Hamilton et al. 2006) used a similar approach to characterise human endometrial biopsies of unknown cycle stage. To identify genes regulated by oestrogens, Andrade and co-workers (Andrade, Silva et al. 2002) treated ovariectomised virgin rats either with conjugated equine oestrogens or placebo and compared endometrial mRNA profiles. Genes regulated by P4 in the ovine uterus resulted from a study with uterine gland knockout ewes (Gray, Abbey et al. 2006).

Bauersachs and co-workers extracted epithelial cells from the bovine oviduct and compared gene expression profiles from the ipsilateral oviduct at oestrus and dioestrus (Bauersachs, Rehfeld et al. 2004). Furthermore they performed a screen of transcriptome changes in bovine intercaruncular endometrium at day 0 (oestrus) and day 12 (dioestrus) of the oestrous cycle. Using a combination of subtracted cDNA libraries and cDNA array hybridisation 133 genes were identified, 65 with higher levels at oestrus and 68 at dioestrus. Genes upregulated at oestrus were assigned to Gene Ontology categories like focal adhesion formation, cell motility, cytoskeleton, extracellular matrix, and cell growth. At dioestrus elevated concentrations of mRNAs coding for a variety of metabolic and transporter proteins were identified. Based on searches in pathway databases and the literature several physiological processes and signalling cascades were obtained, e.g. the TGFB signalling pathway and retinoic acid signalling, which are potentially involved in the regulation of changes of the endometrium during the oestrous cycle. Fourteen genes upregulated at oestrus and three genes upregulated at dioestrus were assigned to the TGFB signalling pathway. Target genes of this signalling pathway (collagens, FBLN1, FBLN5, FN1) and inhibitors of TGFB signalling (DCN, THBS1, INHBA, HTRA1) were found at oestrus. At dioestrus, two of the identified genes also have been shown to have inhibitory functions

(MGP, TDGF1). From the retinoic acid (RA) signalling pathway, which is supposed to play a role in the regulation of the endometrial differentiation state, several involved genes were identified (oestrus: LGALS1, RARRES1, SERPINH1; dioestrus: CYP26A1, RARRES2, RDHE2, TGM2). The results of this study were also compared with microarray studies done in other species. An overlap of 34 genes was found where about half of the mRNA expression changes for these genes were in the same direction. The relative small overlap particularly with the human studies is in line with the distinct differences in the cycle (oestrous cycle lesser changes than during menstrual cycle) and in the type of implantation of the embryo (invasive versus non-invasive implantation) (Bauersachs, Ulbrich et al. 2005). Other studies of this group were focused on transcriptome changes in the endometrium during the pre-implantation phase. Day 18 of pregnancy has been analysed using two different experimental models: i) a comparison of pregnant vs. control endometrium derived from monocygotic twin cows where one twin received an embryo transfer and the corresponding twin a sham transfer and ii) a comparison of endometrium derived from heifers after artificial insemination vs. sham insemination (Bauersachs, Ulbrich et al. 2006; Klein, Bauersachs et al. 2006). Many of the resulting genes are known to be induced by interferon tau, the embryonic pregnancy recognition signal. Based on the identified genes a first model of biological processes and corresponding genes important for embryo implantation and establishment of pregnancy was drawn.

But among all these studies that analysed gene expression changes during the physiological female sexual cycle there was no systematic large-scale gene expression study comparing more than two stages of the cycle. In Publication 2 the custom made cDNA array (BOE array) described in Publication 1 was used for the generation of mRNA expression profiles of bovine endometrium samples derived from several stages of the oestrous cycle: oestrus (day 0), metoestrus (day 3.5), dioestrus (day 12), late dioestrus (slaughtered at day 18, high serum progesterone levels), and preoestrus (slaughtered at day 18, low serum progesterone levels).

4 Publication 1: *Technical Note:* Bovine Oviduct and Endometrium Array Version 1: A Tailored Tool for Studying Bovine Endometrium Biology and Pathophysiology

Stefan Bauersachs, Katrin Mitko, Helmut Blum and Eckhard Wolf

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Stefan Bauersachs: overseeing of the establishment of the BOE array, verifying of the technical reproducibility of the cDNA hybridization, comparison with the existing Affymetrix bovine genome array; authoring of this manuscript

Katrin Mitko: establishing and production of the BOE cDNA microarray

Helmut Blum and Eckhard Wolf: idea; allocation of premises and equipment

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Technical Note: Bovine Oviduct and Endometrium Array Version 1: A Tailored Tool for Studying Bovine Endometrium Biology and Pathophysiology[1]

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ABSTRACT

Fertility problems are the main reason for slaughter of high-performance milk cows, because elongated calving intervals result in financial losses for the farmer and retard genetic progress. Genetic improvement of fertility would be of great benefit, but functional traits for effective selection are missing. Recent advances in functional genomics tools like DNA microarrays could be the key to identify gene expression patterns in the endometrium that correlate with maternal fertility. Therefore, a first version of a bovine oviduct and endometrium cDNA array was established that contains a set of 1,440 cDNA clones and long oligonucleotides representing 950 different genes. The major part of these genes has been identified in a series of differential gene expression studies in endometrium (different stages of the estrous cycle, d 18 pregnant vs. nonpregnant) and in oviduct epithelial cells (different stages of the estrous cycle) using a combination of subtracted cDNA libraries and cDNA array hybridization. Furthermore, cDNA clones of genes, which showed no changes in their mRNA levels in the analyzed tissues, were added as controls. Reproducibility of the array hybridization, a comparison with the Affymetrix bovine genome array, and confirmation of differential gene expression with reverse transcription-quantitative PCR is shown. Potential future applications include systematic studies of interactions between metabolic status and functionality of the endometrium to identify genes that could be used for differential diagnosis of fertility problems. Further, endometrium transcriptome profiles may serve as novel traits to improve fertility by genetic selection.

Key words: functional genomics, endometrium, oviduct, diagnostic array

The endometrium undergoes marked morphological and functional changes during the estrous cycle and during early pregnancy that are mainly regulated by the hormones progesterone, estradiol, and oxytocin (Spencer et al., 2004). To study these changes at the mRNA level, a series of studies using a combination of subtracted cDNA libraries and cDNA array hybridization has been performed to identify differentially expressed genes. In these studies, endometrial samples from heifers at d 0 (estrus) vs. d 12 (diestrus; Bauersachs et al., 2005) and samples from d 12 vs. d 20 (preestrus; unpublished data of S. Bauersachs) have been compared. Day 18 of pregnancy has been analyzed using a monozygotic twin model, in which pregnancy was achieved by transfer of embryos produced in vitro (Klein et al., 2006), and a second model, in which cyclic heifers were inseminated with semen or seminal plasma only and slaughtered on d 18 (Bauersachs et al., 2006). In addition, endometrial samples from d 15 of pregnancy have been compared with d-15 controls (unpublished data of S. Bauersachs). Furthermore, different studies of bovine oviduct epithelial cells during the estrous cycle have been conducted (Bauersachs et al., 2003, 2004). The genes expressed differently in the various studies of endometrial samples and in the oviduct epithelium represent an interesting set of candidates for evaluating the functional state of the female reproductive tract. Therefore, cDNA fragments of all genes identified in the different studies together with cDNA fragments or long oligonucleotides of candidate genes deduced from the literature were integrated on a single cDNA array, the bovine oviduct and endometrium (**BOE**) cDNA array version 1.

More than 20,000 cDNA clones of different subtracted libraries have been screened by microarray analyses in the studies that contributed to the genes on the BOE array. From these clones, 1,344 nonredundant cDNA clones were extracted, mainly consisting of those cDNA

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Table 1. Representation of selected Gene Ontology (http://geneontology.org/) categories on the bovine oviduct and endometrium array

Category	Assigned genes, n
Biological process	
Regulation of transcription, DNA-dependent	88
Immune response	68
Protein biosynthesis	56
Development	45
Cell adhesion	40
Apoptosis	37
Protein AA phosphorylation	36
Proteolysis	35
Cell-cell signaling	33
Metabolism	32
Cell proliferation	26
Cell differentiation	22
Protein transport	22
Cell cycle	21
Intracellular signaling cascade	18
mRNA processing	17
Protein folding	17
Cell surface receptor linked signal transduction	17
Ion transport	17
G-protein-coupled receptor protein signaling pathway	15
Cell motility	14
Ubiquitin cycle	14
Protein AA dephosphorylation	8
Angiogenesis	8
Molecular function	
DNA binding	84
Receptor activity	71
RNA binding	68
Structural molecule activity	45
Transcription factor activity	39
Signal transducer activity	37
Peptidase activity	36
Protein Ser-Thr kinase activity	24
GTPase activity	21
Growth factor activity	20
Endopeptidase inhibitor activity	17
Transcription coactivator activity	14
Sugar binding	13
Translation initiation-elongation factor activity	13
Unfolded protein binding	12
Helicase activity	12
Receptor binding	12
Protein-Tyr kinase activity	11
Cytokine activity	10
Ion channel activity	10
Hormone activity	8
GTPase activator activity	8
IGF binding	8
Steroid hormone receptor activity	7
Cellular component	
Nucleus	183
Cytoplasm	103
Integral to plasma membrane	76
Extracellular region	65
Ribonucleoprotein complex	54
Endoplasmic reticulum	50
Extracellular matrix (sensu Metazoa)	39
Ribosome	39
Cytoskeleton	32
Mitochondrion	30
Cytosol	23
Golgi stack	16
Nucleoplasm	15
Microsome	14
Microtubule	12
Lysosome	11
Heterogeneous nuclear ribonucleoprotein complex	10
Spliceosome complex	9
Collagen	9
Nucleolus	7

Continued

Table 1 (Continued). Representation of selected Gene Ontology (http://geneontology.org/) categories on the bovine oviduct and endometrium array

clones identified as differentially expressed and some control genes, which can be used as internal references. Fragments representing different parts of a transcript were included. Altogether, the BOE array covers approximately 950 different genes. The bovine cDNA clones were annotated based on comparisons to GenBank sequences using the basic local alignment search tool (discontiguous Mega BLAST) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/blast/blast.cgi), searches in the bovine genome (Ensembl; http://www.ensembl.org/Bos_taurus/blastview), and the assignment of corresponding expressed sequence tags to the UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). The human orthologous genes, as far as they exist, were also identified to obtain more information regarding gene function.

Based on the generated list of cDNA clones, the templates for PCR amplification were rearranged from the original 190 to 14 new 96-well plates using a liquid handling station (Multiprobe II Plus EX, Perkin Elmer, Rodgau, Germany). The cDNA fragments were amplified via PCR using modified T7 and T3 primers that bind in the plasmid vector adjacent to the cDNA fragments. The PCR products were spotted on nylon membranes using a microarray robot (Omnigrid Accent, GeneMachines, San Carlos, CA ; Bauersachs et al., 2004). The identity of the cDNA clones was confirmed by DNA sequencing of randomly selected cDNA fragments. Based on the data for the orthologous human genes, a classification according to Gene Ontology (**GO**; http://geneontology.org/) categories was performed. The Affymetrix NetAffx Analysis Center (http://www.affymetrix.com) was used to get a summary of the most specific GO annotations. Data were further reduced to obtain a meaningful overview for the 3 major categories: biological process, molecular function, and cellular component (Table 1). In addition to the GO analysis, the known genes were assigned to molecular pathways (http://www.genome.ad.jp/kegg/tool/search_pathway.html). Table 2 has the numbers of genes that were

Table 2. Assignment of the genes represented on the bovine oviduct and endometrium array to immune-related, adhesion, extracellular remodeling, and signaling pathways

Pathway description	Assigned genes, n
Immune-related pathways	
Cytokine-cytokine receptor interaction	21
Antigen processing and presentation	13
Complement and coagulation cascades	12
Natural killer cell-mediated cytotoxicity	12
Toll-like receptor signaling pathway	11
Jak-STAT[1] signaling pathway	11
Adhesion-remodeling pathways	
Focal adhesion	34
ECM-receptor interaction	19
Regulation of actin cytoskeleton	17
Leukocyte transendothelial migration	16
Gap junction	14
Adherens junction	12
Tight junction	12
Cell adhesion molecules	10
Signaling pathways	
MAPK[2] signaling pathway	25
Cell communication	21
Ca-signaling pathway	13
Neuroactive ligand-receptor interaction	13
Wnt signaling pathway	12
Insulin signaling pathway	11
TGF-β[3] signaling pathway	8
GnRH signaling pathway	8
mTOR[4] signaling pathway	7
Notch signaling pathway	6
Hedgehog signaling pathway	6
VEGF[5] signaling pathway	6
PPAR[6] signaling pathway	5
Other important pathways	
Apoptosis	18
Cell cycle	9

[1]Jak-STAT = Janus kinase signal transducer and activator of transcription.

[2]MAPK = mitogen-activated protein kinase.

[3]TGF = transforming growth factor.

[4]mTOR = mammalian target of rapamycin.

[5]VEGF = vascular endothelial growth factor.

[6]PPAR = peroxisome proliferator-activated receptor.

found in signaling, cell communication and interaction, and immune-related pathways.

Technical reproducibility of the cDNA array hybridization was tested by 3 pairwise hybridizations with cDNA probes derived from the same endometrial tissue sample. The CV of the normalized values (variance stabilization and normalization, BioConductor; Huber et al., 2002) was calculated over the 6 samples and ranged from 0.27 to 11.06% (mean CV 2.11%; median CV 1.86%). Values were not filtered for nondetectable cDNA clones. Analysis with the significance analysis of microarrays (**SAM**) method (Tusher et al., 2001) revealed 2 significantly different cDNA clones with a nominal fold change of 1.4. However, these 2 cDNA clones produced no detectable signals. In conclusion, technical variability was very low and produced no artificial, significantly different hybridization signals (false positives) with the minimal sample size of n = 3.

For a comparison of the BOE array with the existing Affymetrix bovine genome array, which contains approximately 24,000 probe sets, endometrial tissue samples derived from four 18-d pregnant animals (AI) and four 18-d controls were analyzed (for details of sample preparation, see Bauersachs et al., 2006). Raw signals of the BOE array were normalized with vsn (variance stabilization and calibration for microarray data, BioConductor), and differentially expressed genes were identified with the Microsoft Excel add-in SAM (Microsoft Corp., Redmond, WA). Raw data from the Affymetrix arrays were normalized with robust multiarray average (RMA, BioConductor), and significance analysis was done with the SAM tool in siggenes (SAM and Efron's empirical Bayes approaches, BioConductor). With the BOE array, 97 genes with at least 2-fold change were found at a false discovery rate (**FDR**) of 2%. On the Affymetrix array, 352 genes with at least 2-fold change were identified (FDR 2%).

Annotation of differentially expressed probe sets from Affymetrix was completed with Biomart (Ensembl), BLAST (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) analyses, and mapping of probe sets to the bovine genome sequence to allow a comparison with the data from the BOE array. The overlap was 61 genes or 62.9%. All changes of gene expression were consistent between the BOE array and the Affymetrix array. Of the additional 36 genes from the BOE array, 20 were not present on the Affymetrix array based on the bovine Entrez Gene ID of the current annotation (March 2007). The remaining 16 probe sets revealed no significant changes on the Affymetrix array. Sensitivity seems to be similar to that of the Affymetrix array. The large number of additional genes obtained from the Affymetrix array showed mostly changes between 2-fold and 3-fold. Because the cDNA clones of the BOE array are derived from subtracted cDNA libraries, the BOE array is more enriched for genes that show larger gene expression differences.

The expression of 13 genes, which were identified by the BOE array, has been analyzed with reverse transcription-quantitative PCR (**RT-qPCR**) in 2 of our previous studies. The obtained changes in gene expression showed good correlation between RT-qPCR and array data (Table 3).

Although there are already a couple of bovine cDNA arrays (Suchyta et al., 2003; Donaldson et al., 2005; Everts et al., 2005; Jensen et al., 2006) and the bovine genome array from Affymetrix is available, the BOE array has the advantage that it is enriched for genes that are differentially expressed during the most important physiological stages of this tissue. Further-

Table 3. Validation of bovine oviduct and endometrium array results by reverse transcription-quantitative PCR (RT-qPCR)

	Fold change, pregnant vs. nonpregnant[1,2]		*P*-value	
Gene symbol	RT-qPCR	BOE array	RT-qPCR	BOE array
AGRN[2]	6.4	2.5	<0.001	0.005
BST2[2]	16.7	32.6	<0.001	<0.001
C17orf27[2]	9.4	5.6	<0.001	<0.001
C1R[1]	4.7	2.3	0.001	0.005
C1S[1]	5.6	2.5	0.003	<0.001
IFITM3[1]	9.8	5.4	<0.001	<0.001
ISG15[1]	185.6	89.7	<0.001	<0.001
LGALS9[2]	8.1	3.3	<0.001	<0.001
SERPING1[1]	5.2	2.1	0.004	0.008
STAT1[2]	9.2	4.5	<0.001	<0.001
UBE1L[1]	25.6	13.5	<0.001	<0.001
UTMP[1]	163.9	7.4	0.012	<0.001
XAF1[1]	12.1	7.8	<0.001	<0.001

[1]See Klein et al. (2006) for description.

[2]See Bauersachs et al. (2006) for description.

more, the relatively small number of cDNA on the BOE array circumvents many problems of data evaluation and processing and keeps costs down. The platform is flexible, and newly identified interesting genes can be added at any time.

In conclusion, reproducibility of cDNA array hybridization with the BOE array was demonstrated, results were consistent compared with the Affymetrix bovine genome array, and the results of the method were highly correlated with RT-qPCR data. There are manifold applications for the BOE array in basic and applied research for the analysis of endometrial transcriptome changes in normal reproductive physiology and in pathological conditions.

REFERENCES

Bauersachs, S., H. Blum, S. Mallok, H. Wenigerkind, S. Rief, K. Prelle, and E. Wolf. 2003. Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: A transcriptomics approach. Biol. Reprod. 68:1170–1177.

Bauersachs, S., S. Rehfeld, S. E. Ulbrich, S. Mallok, K. Prelle, H. Wenigerkind, R. Einspanier, H. Blum, and E. Wolf. 2004. Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. J. Mol. Endocrinol. 32:449–466.

Bauersachs, S., S. E. Ulbrich, K. Gross, S. E. Schmidt, H. H. Meyer, R. Einspanier, H. Wenigerkind, M. Vermehren, H. Blum, F. Sinowatz, and E. Wolf. 2005. Gene expression profiling of bovine endometrium during the oestrous cycle: Detection of molecular pathways involved in functional changes. J. Mol. Endocrinol. 34:889–908.

Bauersachs, S., S. E. Ulbrich, K. Gross, S. E. Schmidt, H. H. Meyer, H. Wenigerkind, M. Vermehren, F. Sinowatz, H. Blum, and E. Wolf. 2006. Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. Reproduction 132:319–331.

Donaldson, L., T. Vuocolo, C. Gray, Y. Strandberg, A. Reverter, S. McWilliam, Y. Wang, K. Byrne, and R. Tellam. 2005. Construction and validation of a bovine innate immune microarray. BMC Genomics 6:135.

Everts, R. E., M. R. Band, Z. L. Liu, C. G. Kumar, L. Liu, J. J. Loor, R. Oliveira, and H. A. Lewin. 2005. A 7872 cDNA microarray and its use in bovine functional genomics. Vet. Immunol. Immunopathol. 105:235–245.

Huber, W., A. von Heydebreck, H. Sultmann, A. Poustka, and M. Vingron. 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18(Suppl 1):S96–S104.

Jensen, K., R. Talbot, E. Paxton, D. Waddington, and E. J. Glass. 2006. Development and validation of a bovine macrophage specific cDNA microarray. BMC Genomics 7:224.

Klein, C., S. Bauersachs, S. E. Ulbrich, R. Einspanier, H. H. Meyer, S. E. Schmidt, H. D. Reichenbach, M. Vermehren, F. Sinowatz, H. Blum, and E. Wolf. 2006. Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. Biol. Reprod. 74:253–264.

Spencer, T. E., G. A. Johnson, R. C. Burghardt, and F. W. Bazer. 2004. Progesterone and placental hormone actions on the uterus: Insights from domestic animals. Biol. Reprod. 71:2–10.

Suchyta, S. P., S. Sipkovsky, R. Kruska, A. Jeffers, A. McNulty, M. J. Coussens, R. J. Tempelman, R. G. Halgren, P. M. Saama, D. E. Bauman, Y. R. Boisclair, J. L. Burton, R. J. Collier, E. J. DePeters, T. A. Ferris, M. C. Lucy, M. A. McGuire, J. F. Medrano, T. R. Overton, T. P. Smith, G. W. Smith, T. S. Sonstegard, J. N. Spain, D. E. Spiers, J. Yao, and P. M. Coussens. 2003. Development and testing of a high-density cDNA microarray resource for cattle. Physiol. Genomics 15:158–164.

Tusher, V. G., R. Tibshirani, and G. Chu. 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA 98:5116–5121.

5 Publication 2: Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle

Katrin Mitko, Susanne E Ulbrich, Hendrik Wenigerkind, Fred Sinowatz, Helmut Blum, Eckhard Wolf, and Stefan Bauersachs

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Katrin Mitko: Sampling of endometrial tissue samples; isolation of RNA; cDNA synthesis; production of the BOE array; cDNA array hybridisation; array evaluation; quality control using a distance matrix and a heatmap; significance analysis using SAM; hierarchical clustering of genes and samples using HCL support trees; cluster analysis for identification of similar expression profiles; deposition of the data in NCBI's Gene Expression Omnibus (GEO); building of interaction networks; authoring of this manuscript

Susanne E. Ulbrich: quantitative real-time RT-PCR

Hendrik Wenigerkind: animal management

Fred Sinowatz: in situ hybridisation

Helmut Blum and Eckhard Wolf: idea; allocation of premises and equipment

Stefan Bauersachs: overseeing of the analyses; quality control using a distance matrix and a heatmap; deposition of the data in NCBI's Gene Expression Omnibus (GEO); hierarchical clustering of genes and samples using HCL support trees; cluster analysis for identification of similar expression profiles; identifying of quantitatively enriched GO categories using GOTM and OntoExpress; integrated analysis of different functional databases using the 'functional annotation clustering' tool of the DAVID; assigning of the genes to KEGG pathways; building of interaction networks

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Dynamic changes in messenger RNA profiles of bovine endometrium during the oestrous cycle

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Abstract

During the oestrous cycle, the bovine endometrium exhibits characteristic morphological and functional changes, which are mainly induced by progesterone (P_4), oestrogens and oxytocin. We studied the response of the endometrium to this changing hormonal environment at the transcriptome level using a custom-made cDNA microarray. Endometrium samples were recovered from Simmental heifers on days 0 (oestrus), 3.5 (metoestrus), 12 (dioestrus) and 18. The latter group was divided into animals with high (late dioestrus) and low P_4 levels (preoestrus). Significance analysis of microarrays revealed 269 genes exhibiting significant changes in their transcript levels during the oestrous cycle in distinct temporal patterns. Two major types of expression profiles were observed, which showed the highest mRNA levels during the oestrus phase or the highest levels during the luteal phase respectively. A minor group of genes exhibited the highest mRNA levels on day 3.5. Gene ontology (GO) analyses revealed GO categories related to extracellular matrix remodelling, transport, and cell growth and morphogenesis enriched at oestrus, whereas immune response and particular metabolic pathways were overrepresented at dioestrus. Generation of gene interaction networks uncovered the genes possibly involved in endometrial remodelling (e.g. collagen genes, *TNC*, *SPARC*, *MMP2*, *MEP1B*, *TIMP1*, *TIMP2*, *HTRA1*), regulation of angiogenesis (e.g. *ANGPTL2*, *TEK*, *NPY*, *AGT*, *EPAS1*, *KLF5*), regulation of invasive growth (e.g. *PCSK5*, tight junction proteins, *GRP*, *LGALS1*, *ANXA2*, *NOV*, *PLAT*, *MET*, *TDGF1*, *CST6*, *ITGB4*), cell adhesion (e.g. *MUC16*, *LGALS3BP*) and embryo feeding (e.g. *SLC1A1*, *SLC11A2*, *SLC16A1*, *SEPP1*, *ENPP1*). Localisation of mRNA expression in the endometrium was analysed for *CLDN4*, *CLDN10*, *TJP1*, *PCSK5*, *MAGED1*, and *LGALS1*.

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Introduction

Throughout the oestrous cycle and during pregnancy, the bovine endometrium exhibits characteristic morphological and functional changes, which are mainly regulated by progesterone (P_4), oestrogens and oxytocin (Spencer *et al.* 2004*b*). With respect to the typical characteristics of the endometrial structure and function, the bovine oestrous cycle can be divided into four stages: preoestrus, oestrus, metoestrus and dioestrus. At oestrus, P_4 levels are low, whereas oestrogens are elevated. This leads to increased blood circulation and oedematisation of the mucosa and to a high contractility of the smooth musculature. The highly cuboidal endometrial epithelium consists of both ciliated cells and secretory cells, which produce oestral mucus. The uterine glands are elongated as a result of the mucosal oedema (Ruesse & Sinowatz 1998). Oestradiol levels start to decrease just before the luteinising hormone (LH) surge and ovulation. Following ovulation, P_4 levels increase during the 3-day period of metoestrus due to the formation of the new corpus luteum. This phase is characterised by low levels of both P_4 and oestradiol in the peripheral blood. Mucosal oedema and

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contractility of the smooth musculature are declining during metoestrus. During dioestrus, there are high P_4 and low oestradiol levels and the surface epithelial cells are flat. The highly active and proliferating uterine glands secrete uterine milk or histotroph. Its best characterised constituent is uteroferrin, an iron-containing acidic phosphatase, which is involved in the transport of iron to the conceptus. A group of basic protease inhibitors, which have considerable sequence homology to bovine pancreatic trypsin inhibitor (aprotinin), are supposed to control intrauterine proteolytic events initiated by the conceptus. Another secreted basic protein is lysozyme, which is presumed to have an antibacterial effect (Roberts & Bazer 1988). If no embryo is present, luteolytic pulses of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) lead to the regression of the ovarian corpus luteum after day 18 resulting in a strong decrease in P_4 during preoestrus and the entry into a new ovarian cycle (Goff 2004). In contrast, during early pregnancy, the bovine embryo produces interferon-τ (IFNT) as main anti-luteolytic pregnancy recognition signal that prevents luteolysis and the corpus luteum continues production of P_4 as a prerequisite for further development and implantation of the conceptus (Spencer *et al.* 2004*a*).

There are a number of studies using microarrays for the investigation of gene expression changes in the endometrium during the sexual cycle in humans (Horcajadas *et al.* 2007), rhesus monkeys (Ace & Okulicz 2004) and mice (Tan *et al.* 2003). Furthermore, in a first transcriptome study of bovine endometrium during the oestrous cycle, more than 130 genes have been identified as differentially expressed between oestrus and dioestrus (Bauersachs *et al.* 2005). P_4-induced genes have been characterised in sheep endometrium in a study where cyclic ewes were treated with P_4 and endometrial tissue samples were analysed using a custom ovine cDNA array (Gray *et al.* 2006). However, there are no systematic large-scale gene expression studies comparing more than two stages of the sexual cycle. Only recently, human endometrial biopsy samples were collected during the cycle and analysed in a microarray study with the objective to assign the samples to specific stages of the cycle based on their relative mRNA expression profiles (Ponnampalam *et al.* 2004).

In the present study, a custom-made bovine oviduct and endometrium (BOE) cDNA array (Bauersachs *et al.* 2007) was used to generate mRNA expression profiles of the bovine endometrium during the oestrous cycle. Molecular pathways were identified as related to the biological functions of the endometrium in the context of recognition and establishment of early pregnancy.

Results

The animals slaughtered during the oestrous cycle were subdivided into five groups, each with four animals: days 0, 3.5, 12 and 18 with high P_4 levels (>2.8 ng/ml; 18P4H), and day 18 with low P_4 levels (<0.7 ng/ml; 18P4L). To analyse gene expression changes in the bovine intercaruncular endometrium during the oestrus cycle at the level of the mRNA, a custom-made cDNA array (BOE array) was used (Bauersachs *et al.* 2007). This array contains 1344 cDNA fragments and 96 oligonucleotides representing 950 different genes. Most of them were identified as differentially expressed in the endometrium during early pregnancy and the oestrous cycle and in oviduct epithelial cells in several previous studies (Bauersachs *et al.* 2003, 2004, 2005, 2006, Klein *et al.* 2006). RNA of the intercaruncular endometrial tissue samples, derived from the middle part of the ipsilateral uterine horn, was used for the preparation of ^{33}P-labelled probes. In total, 20 BOE arrays were hybridised and evaluated. Quality control (distance matrix and heatmap; BioConductor package geneplotter) of normalised data revealed one hybridisation as an outlier leading to the exclusion of one day 12 sample from the analyses of microarray results. For the identification of mRNAs with significant changes of their concentration in the endometrium during the oestrous cycle, normalised data were analysed with the 'significance analysis of microarrays method (SAM, Multiclass, false discovery rate (FDR) 1%; Tusher *et al.* 2001).

This analysis revealed 427 cDNA fragments or oligonucleotides with significant changes of hybridisation signals during the oestrous cycle. These fragments corresponded to 269 different genes, 267 of them with known or inferred function (Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental). For some cDNAs, no bovine mRNA sequence was found in the GenBank, but the putative human orthologous gene was identified. Two cDNAs could not be assigned to any sequence in the GenBank database and to the bovine genome sequence.

Data from the significant genes were used for cluster analyses. First, data of 19 samples were clustered according to the genes and samples to characterise the differences between the analysed stages of the cycle (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental). The samples clustered in three major groups, consisting of day 0 and day 18P4L (Supplementary Figure 1, left), day 3.5 (middle) and day 12 and day 18P4H animals (right) respectively. The third group split into two subgroups corresponding to the day 12 and day 18P4H animals. The day 18P4L and day 0 animals did not split into two distinct separate groups. A two-class SAM analysis for days 0 and 18P4L revealed only two transcripts, which were significantly more abundant in the 18P4L animals. The cluster analysis in Supplementary Figure 1 shows that the greatest difference is present between the animals of the oestrous (days 18P4L and 0) and dioestrus stages (day 12).

For the identification of groups of genes showing similar dynamic patterns in their expression levels during the oestrous cycle, a second cluster analysis was performed. Different numbers of clusters from five to ten were tested and the number of seven clusters was found best to

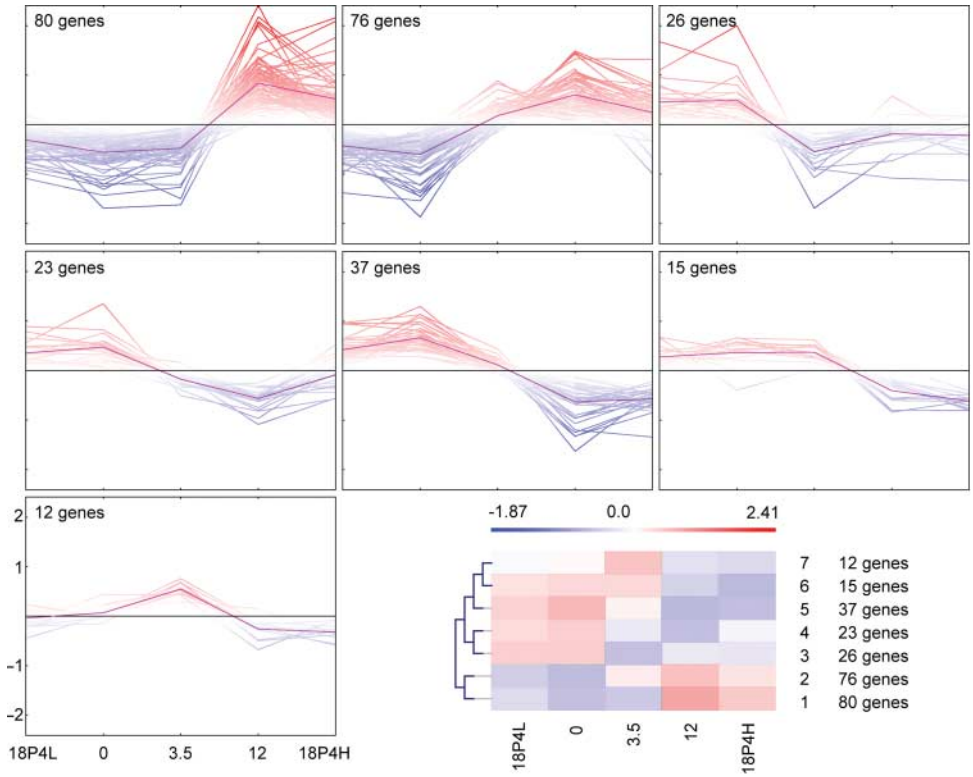

Figure 1 Clusters of similar expression profiles during the oestrous cycle. Genes were clustered according to the correlation of their expression profiles during the oestrous cycle using self-organising tree algorithm (SOTA, MultiExperiment Viewer 4.0). The SOTA dendrogram (bottom right) shows the similarity between clusters.

represent similar expression profiles (Fig. 1). In the first cluster (80 genes), the mRNA levels are low at oestrus (days 18P4L and 0) and metoestrus (day 3.5) and rise on day 12 (dioestrus). Cluster 1 also contains a number of genes with increasing mRNA levels from days 12 to 18P4H but a two-class SAM analysis between the groups, days 12 and 18P4H, revealed almost no significantly higher signals at day 18P4H compared with day 12. In the second cluster (76 genes), the mRNA levels are also low at oestrus (days 18P4L and 0) but are already increased on day 3.5. Clusters 3 (26 genes), 4 (23 genes) and 5 (37 genes) have all their highest expression levels at days 18P4L and 0 (oestrus stage). Cluster 3 differs from 4 and 5 therein that the mRNA levels are decreased at days 3.5, 12 and 18P4H with the lowest levels at day 3.5. Clusters 4 and 5 look quite similar, but in cluster 4 mRNA levels are lower at day 3.5 compared with cluster 5 and start to increase at day 18P4H. Cluster 6 (15 genes) contains genes that have higher mRNA levels from days 18P4L to 3.5 (oestrus to metoestrus) and low levels at days 12 and 18P4H. Cluster 7 consists of 12 genes with the highest mRNA levels at day 3.5. However, some genes, which have also their highest expression at day 3.5, were assigned to the second cluster since – in contrast to cluster 7 – their mRNA concentrations are higher at dioestrus compared with oestrus.

Ninety-four of the genes showed their highest mRNA expression level in the groups, days 18P4L and 0; 23 genes at day 3.5; 112 genes at day 12; and 40 genes at day 18P4H.

The results of the cDNA array hybridisation were verified and absolutely quantified by the use of quantitative real-time RT-PCR (qPCR). The same RNA samples as for the array hybridisation were used. Eleven genes were selected for the validation of different types of mRNA expression profiles revealed by the microarray analysis. Furthermore, most of the genes were chosen due to their proposed functions in the context of maternal fertility and establishment of pregnancy. Table 1 shows the relative expression data obtained by qPCR and the corresponding array data. The table shows mean fold changes between various stages of the oestrous cycle when compared with the stage with the lowest expression (set to 1.0). Array and qPCR data correlated well. However, two of the analysed mRNAs showed no significant ($P<0.05$) changes according to the qPCR.

For the analysis of gene ontology (GO) classifications and pathway analyses, clusters 1 and 2 (high expression levels at dioestrus) and clusters 3, 4, 5 and 6 (high expression levels at oestrus) were combined respectively. Enriched and underrepresented GO categories (significance threshold: ≥ 3 genes, $P \leq 0.01$) were identified using GO tree machine (GOTM) and OntoExpress respectively. Analyses were done based on Entrez Gene IDs of the putative human orthologous genes. Redundant and unspecific categories were removed. Enriched GO categories for genes with higher expression at oestrus are shown in Table 2. GO categories related to extracellular matrix (ECM) remodelling (e.g. 'collagen', 'extracellular region', 'ECM'), transport processes (e.g. 'transport', 'intracellular transport', 'establishment of protein localisation', 'endoplasmic reticulum', 'cytoskeleton-dependent

Table 1 Validation of array results using real-time RT-PCR.

Gene	Gene name	Analysis	Day 18P4L	Day 0	Day 3.5	Day 12	Day 18P4H	*P* value*
CLDN10	Claudin 10	Array	14.1	14.8	7.2	1.3	1.0	<0.001
		qPCR	28.3	33.9	13.0	1.2	1.0	<0.001
IRF1	Interferon regulatory factor 1	Array	1.5	1.0	2.0	2.5	3.4	0.001
		qPCR	1.3	1.0	1.3	1.2	2.0	0.064
LGALS1	Galectin-1	Array	2.6	4.0	2.1	1.1	1.0	<0.001
		qPCR	3.2	5.2	2.0	1.1	1.0	<0.001
PLA2G10	Phospholipase A2, group X (secreted phospholipase A2)	Array	2.4	1.0	2.1	8.1	3.9	<0.001
		qPCR	2.2	1.0	3.5	5.3	9.7	<0.001
PFN1	Profilin 1	Array	2.2	2.5	1.3	1.0	1.6	0.002
		qPCR	2.7	2.5	1.0	1.0	1.2	0.006
SERPING1	C1 inhibitor	Array	1.9	1.0	1.9	5.8	4.0	<0.001
		qPCR	1.5	1.0	1.7	2.0	3.6	0.008
STC1	Stanniocalcin 1	Array	3.3	3.4	1.0	1.3	1.8	0.001
		qPCR	8.9	4.3	1.0	1.0	2.8	0.001
TDGF1	Teratocarcinoma-derived growth factor 1 (Cripto-1)	Array	1.1	1.0	1.0	28.0	1.4	<0.001
		qPCR	1.6	1.0	1.0	88.8	1.8	<0.001
TNC	Tenascin C (hexabrachion)	Array	10.3	11.8	4.9	1.0	1.7	<0.001
		qPCR	27.0	46.8	14.1	1.0	2.3	<0.001
TJP1	Tight junction protein 1 (zona occludens 1)	Array	1.5	1.0	2.9	3.5	2.2	0.002
		qPCR	1.0	1.0	1.5	1.2	1.1	0.219

**P* value for array data.

intracellular transport'), cell growth and morphogenesis (e.g. 'insulin-like growth factor (IGF) binding', 'regulation of cell growth', 'cell morphogenesis') and cell shape (e.g. 'cytoskeleton organisation and biogenesis', 'cytoskeletal part', 'actin filament', 'microtubule-based movement') were found. Table 3 shows enriched GO categories for genes with higher mRNA levels at dioestrus. Overall, less and completely different enriched categories were found compared with oestrus. The majority of enriched GO categories belonged to the term 'response to stimulus', which comprises subcategories such as 'immune response', 'response to chemical stimulus', 'B-cell-mediated immunity' and 'antigen processing and presentation of endogenous antigen'. Furthermore, a number of distinct metabolism categories (e.g. 'lipid catabolism', 'xenobiotic metabolic process', 'monooxygenase activity', 'TCA cycle') were obtained. In addition to the GO analysis, the KEGG pathway database was searched for the identified genes. Genes with higher mRNA levels at oestrus were assigned to 42 pathways and those with higher expression levels at dioestrus to 103 pathways. Considering only pathways where at least two genes were assigned, 14 were obtained at oestrus and 43 at dioestrus. The highest numbers of genes with increased mRNA levels at oestrus were assigned to the pathways 'focal adhesion' (nine genes), 'cell communication' (eight

Table 2 Quantitatively enriched gene ontology (GO) categories for genes up-regulated at oestrus (day 0/day 18P4L).

GO category	Genes	*P* value	Tool
Extracellular region	29	<0.001	OntoExpr[a]
Transport	28	<0.001	GOTM[b]
Extracellular matrix	13	<0.001	GOTM
Intracellular transport	13	<0.001	GOTM
Establishment of protein localisation	10	0.005	GOTM
Endoplasmic reticulum	10	0.008	OntoExpr
Cytoskeletal part	9	0.005	GOTM
Regulation of cell growth	8	<0.001	OntoExpr
Cell morphogenesis	8	0.001	GOTM
Cytoskeleton organisation and biogenesis	8	0.003	GOTM
IGF binding	5	<0.001	GOTM
Cytoskeleton-dependent intracellular transport	5	<0.001	GOTM
Growth factor activity	5	0.004	OntoExpr
Microtubule-based movement	4	<0.001	OntoExpr
Microsome	4	0.005	OntoExpr
Protein disulphide isomerase activity	3	<0.001	GOTM
Actin filament	3	0.001	GOTM
Neuropeptide signalling pathway	3	0.003	OntoExpr
Positive regulation of I-κB kinase/NF-κB cascade	3	0.005	OntoExpr

[a]OntoExpress. [b]GO tree machine.

Table 3 Quantitatively enriched gene ontology (GO) categories for genes up-regulated at dioestrus (day 12/day 18P4H).

GO category	Genes	*P* value	Tool
Response to stimulus	45	<0.001	OntoExpr[a]
Immune response	18	<0.001	OntoExpr
Response to chemical stimulus	12	0.005	OntoExpr
MAPKKK cascade	8	<0.001	OntoExpr
Lysosome	8	<0.001	GOTM[b]
Endopeptidase inhibitor activity	6	0.005	GOTM
Lipid catabolism	5	0.004	GOTM
Microsome	5	0.008	OntoExpr
Xenobiotic metabolic process	4	<0.001	OntoExpr
B-cell-mediated immunity	4	0.002	GOTM
Monooxygenase activity	4	0.006	OntoExpr
Oxygen and reactive oxygen species metabolic process	4	0.007	OntoExpr
Helicase activity	4	0.008	OntoExpr
Aromatic compound metabolic process	4	0.011	OntoExpr
Antigen processing and presentation of endogenous antigen	3	0.001	GOTM
Tricarboxylic acid cycle	3	0.002	GOTM
Mitochondrial matrix	3	0.005	OntoExpr

[a]OntoExpress. [b]GO tree machine.

genes), and 'ECM–receptor interaction' (eight genes). The genes assigned to these pathways were almost identical with six of them coding for collagens. Three tubulin genes (*TUBB*, *TUBA1*, *TUBA1B*) were assigned to the gap junction pathway. Pathways with the highest numbers of assigned genes showing higher mRNA levels at dioestrus were 'focal adhesion' (five genes) and with four assigned genes respectively three metabolic pathways ('citrate cycle', 'reductive carboxylate cycle', 'starch and sucrose metabolism'), 'complement and coagulation cascades' and 'antigen processing and presentation'. Figure 2 shows pathways that contain genes of both types of regulation.

The genes with elevated mRNA levels either at oestrus or at dioestrus were further analysed with the 'functional annotation clustering' tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID; Dennis *et al.* 2003). The results of this integrated analysis of different functional databases (e.g. GO, KEGG pathways, SP-PIR keywords) are clusters of more or less enriched members with overlapping sets of genes, thus resulting in a significant cluster enrichment score (geometric mean in − log scale of member's Fisher exact *P* values in a corresponding annotation cluster). An enrichment score of 1.3 corresponds to a *P* value of 0.05. The results of this analysis are summarised in Tables 4 and 5, where the most informative terms, the number of genes assigned to the cluster and the enrichment score of the cluster are shown. At oestrus, most significant clusters are containing members related to extracellular region, focal adhesion, transport, morphogenesis, regulation of cell growth, protein transport and cytoskeleton. At dioestrus, the highest enrichment scores were found for response to stimulus, immune response, glycoprotein, lysosome, transcription factor activity and carbohydrate metabolism.

Using Pathway Architect software (version 2.0.1, Stratagene, Heidelberg, Germany), interaction networks were built for genes up-regulated at oestrus (self-organising tree algorithm, SOTA clusters 3, 4, 5, and 6 in Fig. 1) and genes up-regulated at dioestrus (SOTA clusters 1 and 2 in Fig. 1) respectively. Networks were drawn based on the different types of interactions like binding, regulation, expression, transport, assignment to a protein family or a biological process. Furthermore, interactions with small molecules and proteins not regulated, but substantially contributing to the network, were also included. Proteins were assigned to their primary cellular localisation. Figure 3 (see also Supplement to Figure 3, which is available online at www.reproduction-online/supplemental/) shows the interaction network for the genes with higher mRNA concentrations at oestrus, of which 67 could be assigned. In Fig. 4 (see also Supplement to Figure 4, which is available online at www.reproduction-online/supplemental/), the network of genes with elevated mRNA levels at dioestrus (103 genes assigned) is shown. For further details on the interactions and the genes, see the supplemental material (navigable HTML files).

For six selected genes (claudin 10 (*CLDN10*), *CLDN4*, galectin-1 (*LGALS1*), melanoma antigen family D, 1 (*MAGED1*), prohormone convertase 5 (*PCSK5*) and tight junction protein 1 (*TJP1*)), *in situ* hybridisation with the bovine endometrial tissue sections corresponding to those used for microarray analyses was carried out (Fig. 5). These six genes were selected due to their potential role in cell adhesion and regulation of implantation. As revealed by the array hybridisation, *CLDN10*, *LGALS1*, *MAGED1* and *PCSK5* mRNAs showed the highest levels at oestrus, *TJP1* at day 12 and *CLDN4* at day 18 (P4 high). A specific pattern of mRNA distribution in the endometrium was found for each of these genes. The hybridisation signal was always confined to the cells of the endometrium. No mRNA staining was seen in the myometrium or the serosa. No specific signal was observed in the sections hybridised with the sense strand or in sections that were incubated with buffer only instead of the oligoprobes. Table 6 summarises the results of the mRNA localisation experiments.

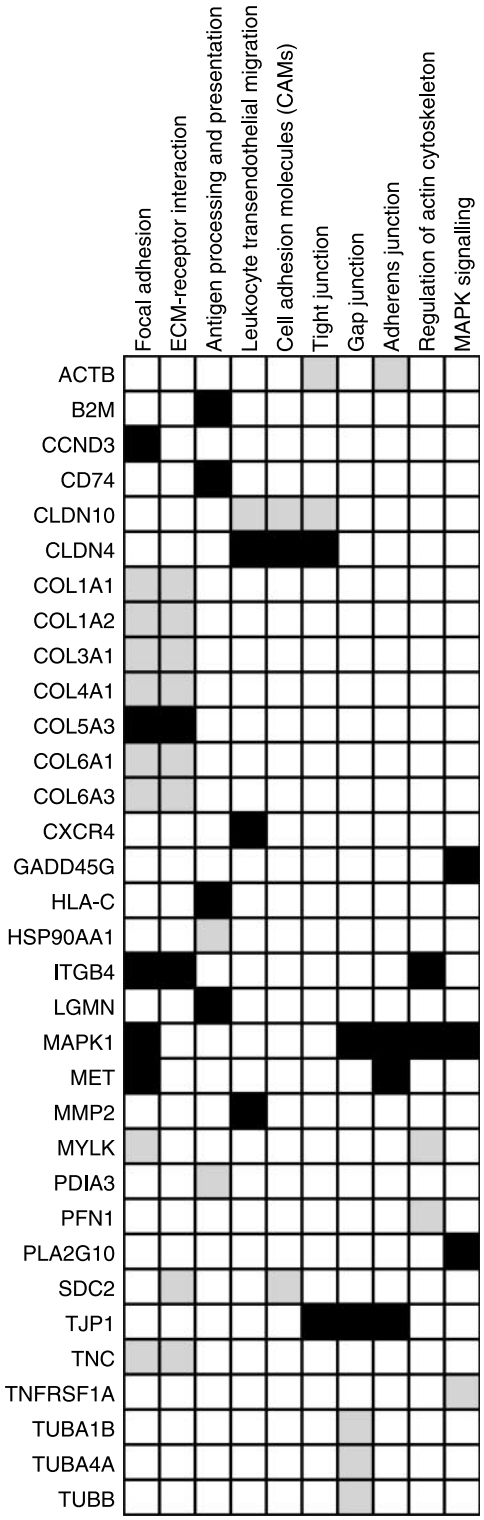

Figure 2 Results of KEGG pathway search. KEGG pathways were searched for the differentially expressed genes. Pathways containing both genes with the highest mRNA levels at oestrus and genes with the highest mRNA levels at dioestrus are shown. Grey boxes, genes up-regulated at oestrus; black boxes, genes up-regulated at dioestrus.

Discussion

After an initial investigation of the differential gene expression in the bovine endometrium between the oestrus and dioestrus stages in our previous study (Bauersachs *et al.* 2005), the present work provides the first study of dynamic changes in the mRNA expression profiles between several time points of the oestrous cycle. As in our earlier studies, the intercaruncular endometrium was used for gene expression analyses, since there are distinct differences between the caruncular and intercaruncular endometrium regarding morphology and function. Most notably, the caruncles contain no uterine glands. After embryo implantation, the caruncles grow rapidly and form individual placentomes, which are responsible for gaseous and small molecule exchange. Because the uterine glands play an important role in the supply of large molecules to the embryo before and after implantation (Atkinson *et al.* 1984), the intercaruncular tissue samples were preferred for gene expression analyses.

For the oestrous stage, two groups of samples were collected corresponding to early oestrus (around oestradiol peak) and late oestrus (after LH surge). Furthermore, the early luteal phase, the dioestrus phase and the late luteal phase were included. The latter cycle stage was analysed because the implantation of the bovine embryo is initiated at day 18, which requires appropriate conditioning of the endometrium (Bauersachs *et al.* 2006, Klein *et al.* 2006).

The statistical analysis of the microarray data as well as the hierarchical clustering hardly showed any significant differences between the early (18P4L) and the late oestrus samples (day 0). Either no major differences in the mRNA levels between the early and late oestrus exist or the day 0 and day 18P4L groups were not homogeneous enough regarding their hormone levels to detect significant differences in the endometrial transcriptome profiles. Inter-individual variation is clearly an important issue, since genes like *TGFB1* (transforming growth factor β1) show only transient mRNA up-regulation for a few hours after oestrogen induction (Takahashi *et al.* 1994). The most pronounced differences were found between the oestrus and dioestrus stages. The gene expression at day 3.5 is an intermediate between oestrus and dioestrus for most of the analysed genes either increasing or decreasing during the luteal phase. In addition to the marked differences between oestrus and dioestrus, significant differences were found between the mid- and late luteal phase with the mRNA levels mainly decreasing during the late luteal phase in correlation with lower P_4 levels in the day 18P4H group compared with the day 12 group.

The analysis of GO categories, KEGG pathways and functional annotation clustering revealed quantitatively enriched biological processes and molecular functions, which are distinct for the ovulatory and the luteal phases. In accordance with our previous results (Bauersachs *et al.*

Table 4 Results of functional annotation clustering for genes with higher mRNA levels at oestrus.

Cluster description	Genes	Score
Signal peptide/glycoprotein/extracellular region	40	6.13
Structural molecule activity/extracellular matrix/coiled coil/focal adhesion/cell communication	38	4.09
Morphogenesis/regulation of cell growth/IGF binding	12	3.74
Cytoplasm/transport	40	2.41
Acetylation/cytoskeleton/cytoskeletal protein binding	18	2.04
Transporter activity/generation of precursor metabolites and energy/thioredoxin fold/protein disulphide isomerase activity	12	2.01
Establishment of protein localisation/endoplasmic reticulum	13	1.80
Cell organisation and biogenesis/purine nucleotide binding/cytoskeleton/hydrolase activity	39	1.71
Cell motility	5	1.32
G-protein-coupled receptor protein signalling pathway/neuropeptide	5	1.23

2005), a number of mRNAs coding for the ECM proteins and the components of the cytoskeleton were found as enriched at oestrus, indicating a remodelling of the ECM in the endometrium and changes of the cytoskeleton of endometrial cells. The lower mRNA levels of collagen genes at dioestrus are in line with the results of a recent study (Yamada *et al*. 2002) where a decrease in collagen fibres has been observed during the pre-implantation phase in the bovine endometrium. This also fits well with the expression profiles of *MMP2* (SOTA cluster 2) and *MEP1B* (SOTA cluster 1) mRNAs coding for proteases involved in the ECM degradation (Kaushal *et al*. 1994). *MEP1B* mRNA showed the highest levels at days 12 and 18. The *MMP2* mRNA expression profile was not typical for cluster 2, since concentration was the highest at day 3.5 and low at all other analysed time points. The expression profiles of *TIMP1* (cluster 3) and *TIMP2* (cluster 1) mRNAs, coding for inhibitors of matrix metalloproteinases, were opposite to *MMP2* with the lowest levels at day 3.5. The mRNA levels of most collagen genes and other constituents of the ECM were similar from days 18P4L to 3.5 and low at days 12 and 18 (clusters 5 and 6). *HTRA1* mRNA codes for a serine protease that is activated by C-propeptides of fibrillar collagens (Murwantoko *et al*. 2004) and has been shown to cleave TGFB1 and other members of the TGFB family (Oka *et al*. 2004). The mRNA expression profile (cluster 6) was similar to those of fibrillar collagens. In addition, the expression of *HTRA1* has been shown in the human placenta at the maternal–trophoblast interface (Nie *et al*. 2006). The mRNA expression profiles of all these genes suggest a complex regulation of the ECM remodelling in the bovine endometrium during the oestrous cycle.

Probably, in connection with increased ECM synthesis, genes relevant for protein folding and secretion were enriched at oestrus. For example, SERPINH1 (serpin peptidase inhibitor, clade H, member 1), also known as HSP47, has been described as collagen-binding protein of the endoplasmic reticulum involved in collagen processing and secretion (Nagata 1996). Furthermore, transcripts of the genes related to the regulation of cell growth were overrepresented at oestrus. These include mRNAs of a number of IGF-binding proteins, which are important for the regulation of cell growth. The interaction network shows that they are probably regulated by TGFB1, retinoic acid (RA) and oestrogen in a complex manner. Oestrogen may mediate a number of its effects on the endometrium via induction of TGFB1 and RA. Direct induction of expression of RA biosynthetic enzymes has been found in the rat uterus (Li *et al*. 2004). In addition, prostaglandin E2α (PGE2a), interferons (IFNG) and tumour necrosis factor-α are suggested as important regulators by the oestrus interaction network. A number of genes with higher mRNA levels at oestrus were found in the context of the process focal adhesion formation. Genes coding for ECM proteins and components of the cytoskeleton, which are linked via paxillin, are involved in this process. A possible candidate for the regulation of cytoskeleton proteins is polo-like kinase 2 (PLK2; SOTA cluster 5), also known as serum-inducible kinase SNK. PLK2 has been shown to play a role in the remodelling of synapses via phosphorylation of spine-associated Rap guanosine

Table 5 Results of functional annotation clustering for genes with higher mRNA levels at dioestrus.

Cluster description	Genes	Score
Response to stimulus/immune response	38	3.40
Glycoprotein/signal peptide	44	3.39
Vacuole/lysosome	8	3.21
Humoral immune response/complement and coagulation cascades	12	1.95
Transcription factor activity/interferon regulatory factor	6	1.69
Catabolism/carbohydrate metabolism/TCA cycle	20	1.62
Response to chemical stimulus/endoplasmic reticulum/monooxygenase	15	1.55
Enzyme regulator activity/endopeptidase inhibitor activity	10	1.52
Immune response/antigen processing and presentation	4	1.49
Generation of precursor metabolites and energy/mitochondrion/TCA cycle	14	1.32

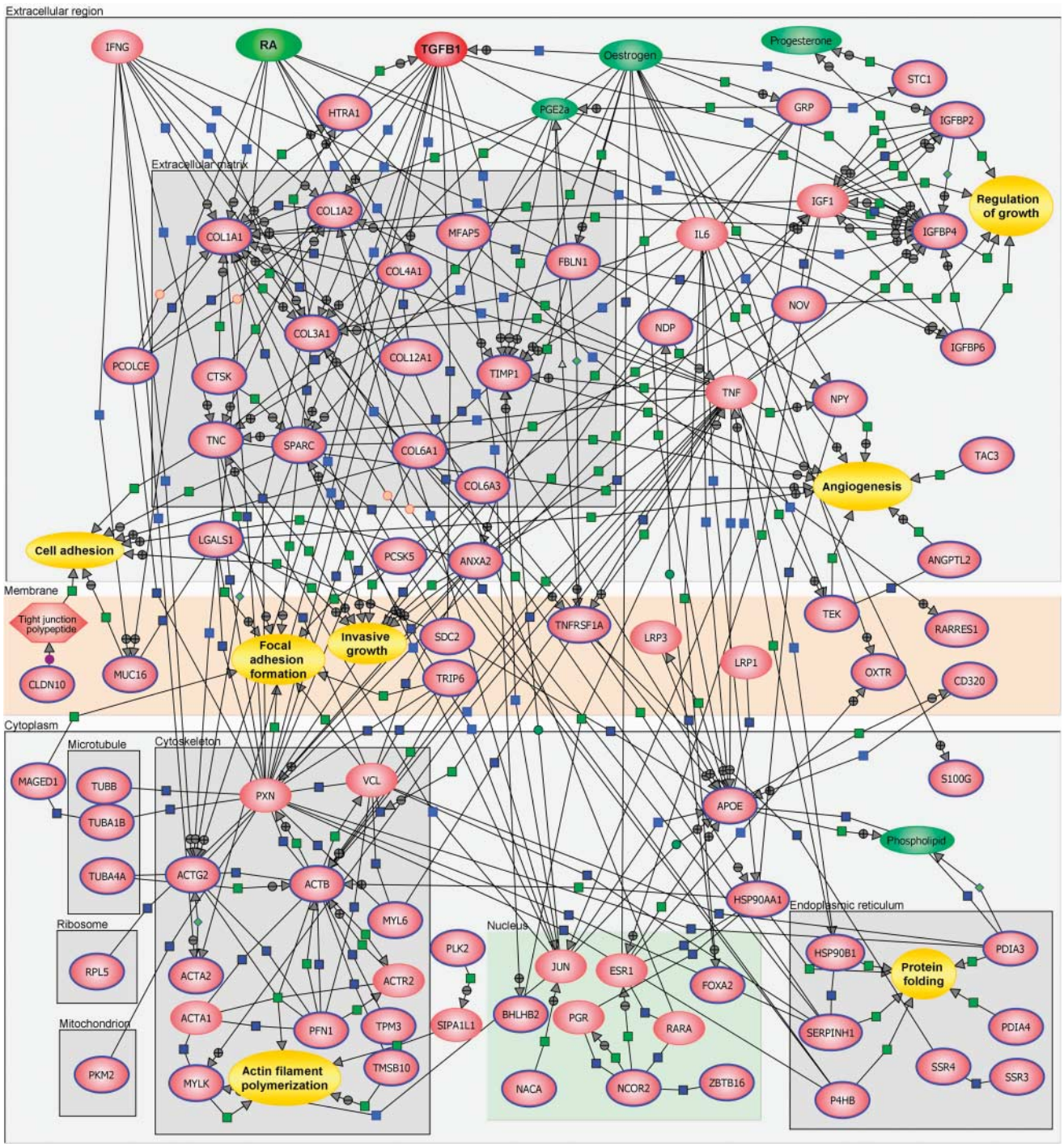

Figure 3 Interaction network of genes with the highest mRNA levels at oestrus. Proteins (red) are assigned to their primary cellular localisations. Proteins with higher mRNA levels at oestrus are highlighted in blue. Small molecules are in green and biological processes in yellow. Interaction types: dark blue squares, binding; light blue squares, expression; green squares, regulation; green circles, promoter binding; orange circles, protein modification; purple circles, member; green triangle, transport; cyan diamond, metabolism. Further information on nodes and interactions can be found in Supplement to Fig. 3 (navigable HTML, which is available online at www.reproduction-online/supplemental/).

triphosphatase-activating protein (SPAR), a postsynaptic actin regulatory protein, leading to the degradation of SPAR (Pak & Sheng 2003). PLK2 could play a similar role in the modulation of actin filament polymerisation in the bovine endometrium. A number of genes such as *LGALS1*, tenascin C (*TNC*), osteonectin (*SPARC*), *PCSK5*, gastrin-releasing peptide, annexin A2 and nephroblastoma overexpressed gene; SOTA clusters 5, 5, 6, 4, 3, 5, 5) have been described as positively involved in the regulation of the process 'invasive growth'. The decreased

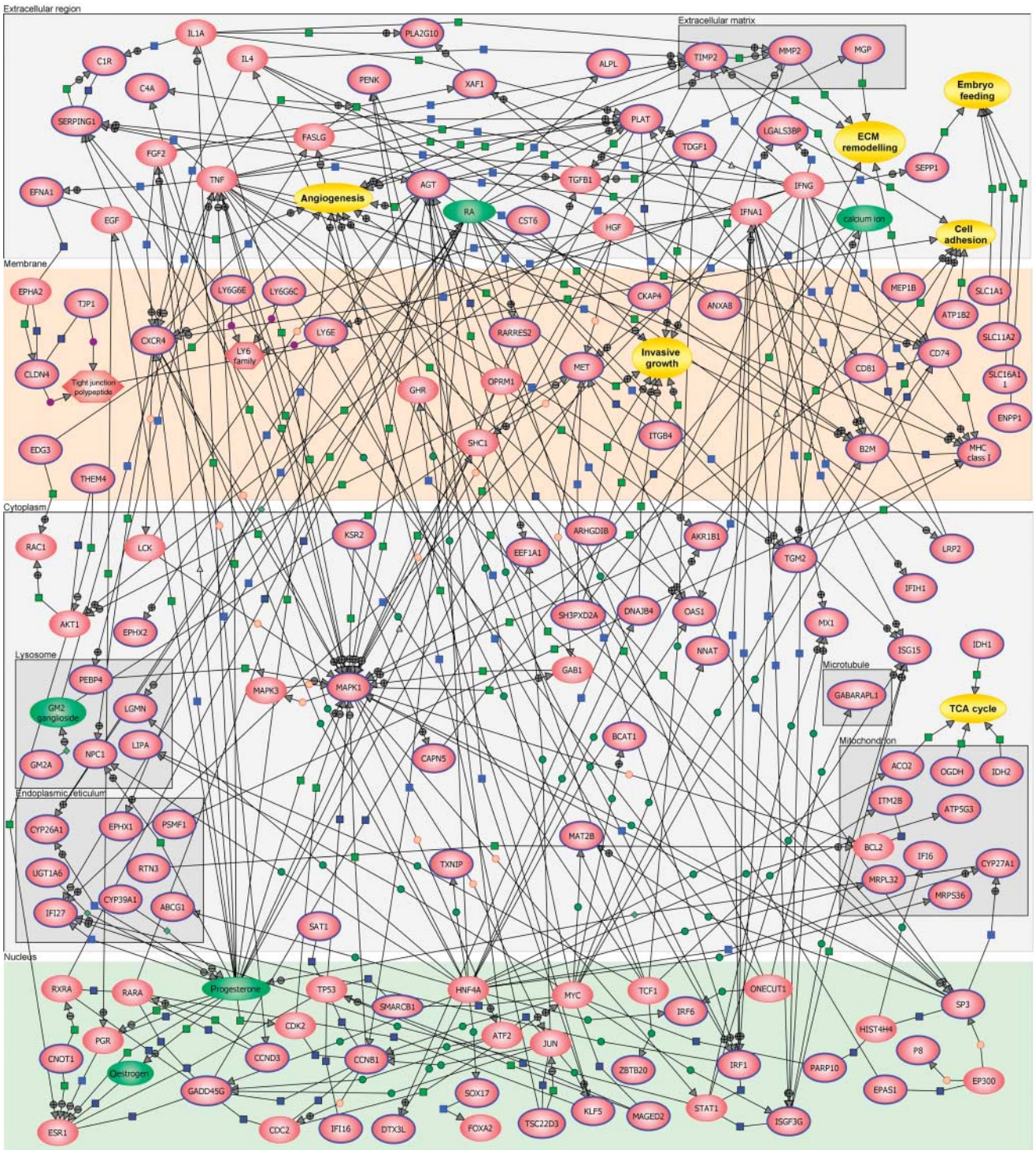

Figure 4 Interaction network of genes with the highest mRNA levels at dioestrus. Proteins (red) are assigned to their primary cellular localisations. Proteins with higher mRNA levels at dioestrus are highlighted in blue. Small molecules are in green and biological processes in yellow. Interaction types: dark blue squares, binding; light blue squares, expression; green squares, regulation; green circles, promoter binding; orange circles, protein modification; purple circles, member; green triangle, transport; cyan diamond, metabolism. Further information on nodes and interactions can be found in Supplement to Fig. 4 (navigable HTML, which is available online at www.reproduction-online/supplemental/).

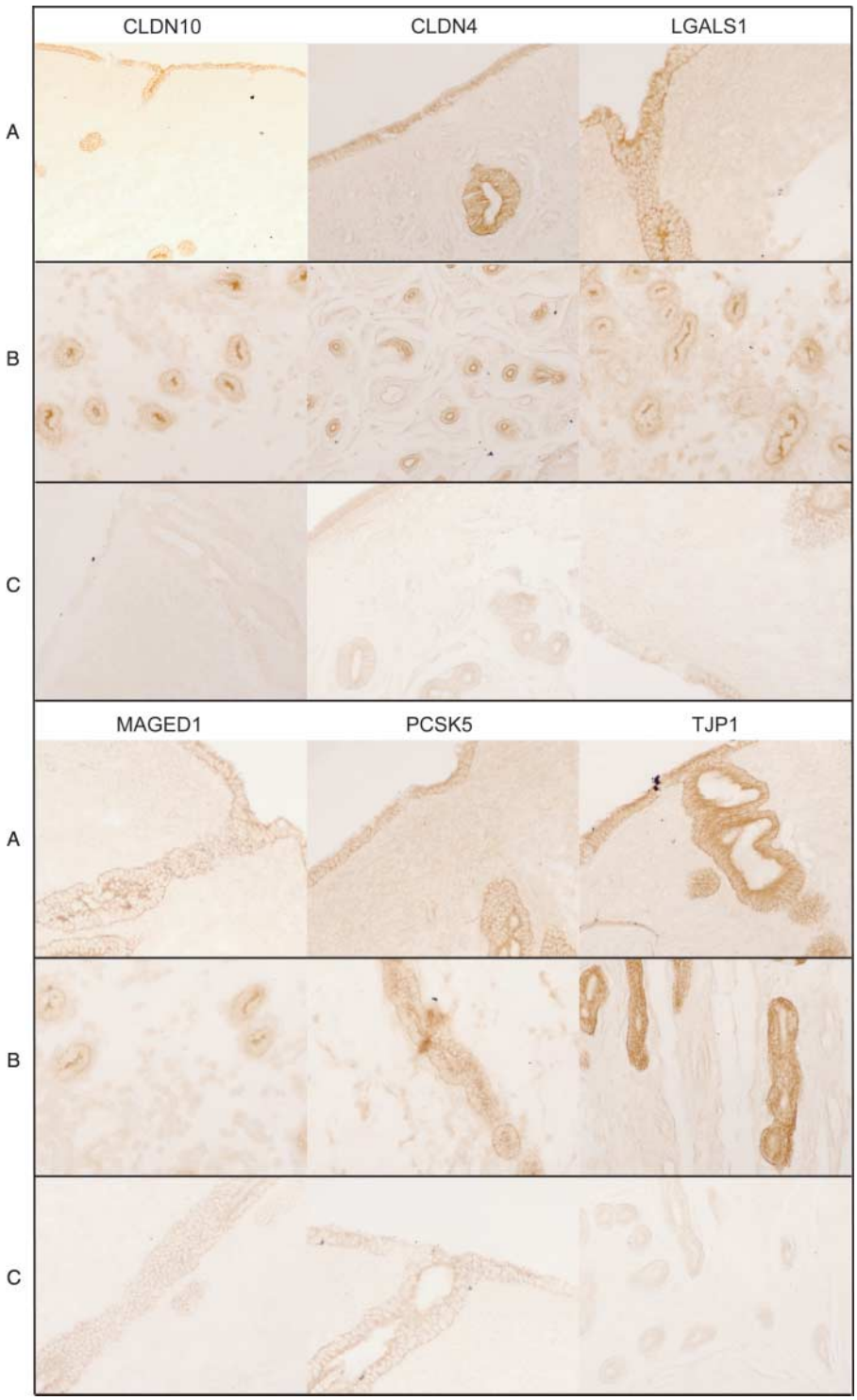

Figure 5 Localisation of mRNA expression of selected genes in the endometrial tissue samples by *in situ* hybridisation. Endometrial tissue samples were obtained from animals slaughtered at different days of oestrous cycle for the detection of *CLDN10*, *LGALS1*, *MAGED1* and *PCSK5* mRNAs at oestrus (day 0) or *CLDN4* and *TJP1* mRNAs at dioestrus (days 18P4H and 12 respectively). Endometrial sections (A) near the epithelial surface, (B) of the deep uterine glands and (C) the corresponding sense controls are shown.

mRNA levels of these genes during the luteal phase may be associated with the regulation of non-invasive implantation in cattle. Furthermore, a couple of genes are involved in the cell adhesion processes. Most importantly, *MUC16* mRNA is down-regulated during the luteal phase (cluster 4). MUC16 protein has been shown to prevent initial trophoblast cell adhesion and is lost in the human endometrium during the receptive phase (Gipson *et al.* 2007).

It has been known for a long time that uterine blood flow substantially changes during the oestrous cycle with increased blood flow during oestrus (Greiss & Anderson 1969). In this context, a number of genes up-regulated at oestrus are linked with the process angiogenesis in the

Table 6 Localisation of mRNA expression of selected mRNAs in endometrial tissue samples by *in situ* hybridisation.

Gene	Gene name	Luminal epithelium	Glandular epithelium		Stromal cells
			Superficial	Deeper glands	
CLDN10	Claudin 10	++	++	+	(+)
CLDN4	Claudin 4	++	+++	+	−
LGALS1	Galectin-1	+	++	+	(+)
MAGED1	Melanoma antigen family D, 1 (NRAGE)	+	+/++	(+)	−
PCSK5	Prohormone convertase 5	+	+	(+)	(+)
TJP	Tight junction protein 1 (zona occludens 1)	+/++	++	+++	(+)

−, no staining; (+), weak staining; +, moderate staining; ++, pronounced staining; +++, strong staining.

interaction network. Some of them are known to directly stimulate angiogenesis. *ANGPTL2* (angiopoietin-like 2; cluster 5) is related to the angiopoietins and has been described to regulate endothelial cell growth (Morisada *et al.* 2006). *TEK* (TEK tyrosine kinase, endothelial; cluster 5) encodes the angiopoietin receptor TIE-2. Neuropeptide Y (cluster 3) is a potent angiogenic factor as well as a stimulator of vascular smooth muscle proliferation (Abe *et al.* 2007).

For the genes up-regulated at dioestrus GO enrichment, analyses revealed predominantly several immunological processes like 'immune response', 'response to stimulus' or 'antigen processing and presentation'. Many of the corresponding genes have been described as interferon induced or were identified in our previous studies of endometrium at day 18 of pregnancy (Bauersachs *et al.* 2006, Klein *et al.* 2006). This suggests that interferons could also play a role in endometrial gene regulation during the oestrous cycle or the corresponding genes are additionally regulated through P_4 effects. However, in a study of porcine endometrium, lymphocytes and other immune cells have been shown to immigrate into the endometrium during dioestrus (Kaeoket *et al.* 2001). In the interaction network of the luteal stage of the oestrous cycle, two of the most connective nodes are MAP kinase 1 (*MAPK1*; cluster 1) and P_4. MAPK1 acts as an interconnection for many cellular processes and is involved in the regulation of translation and gene activation (Rolli-Derkinderen *et al.* 2003). In contrast to the 'oestrus interaction network', much more nuclear factors can be found at dioestrus correlating with the diverse functional classes of genes found as up-regulated at dioestrus.

The process angiogenesis is connected in both the interaction networks with a number of genes that predominantly have been described as positive regulators of this process. Some of them are more related to angiogenesis in the context of tumour progression and invasion, such as *PLAT* (tissue plasminogen activator; cluster 1), *TIMP2*, *MMP2* and *MET* (hepatocyte growth factor receptor; cluster 2; Fridman *et al.* 1992, Diaz *et al.* 2002, Ma *et al.* 2003). Genes with higher mRNA levels during the luteal phase, which are more directly involved in angiogenesis are ephrin-A1 (*EFNA1*), endothelial differentiation sphingolipid G-protein-coupled receptor, 3 (*EDG3*), angiotensinogen (*AGT*), endothelial PAS domain protein 1 (*EPAS1*; hypoxia-inducible factor 2-α) and Kruppel-like factor 5 (*KLF5*; clusters 1, 1, 2, 1, 2). *AGT* codes for the progenitor of angiotensin II, which has been described to function as a vasoconstrictor and to regulate fetoplacental angiogenesis in the ovine placenta (Zheng *et al.* 2005). *EPAS1* and *KLF5* code for transcription factors controlling the expression of vascular endothelial growth factor (*VEGF*), VEGF receptor 1 (*FLT1*), VEGF receptor 2 (*KDR*), and *TEK* (Takeda *et al.* 2004) and of platelet-derived growth factor (*PDGF*), early growth response 1, (*EGR1*), plasminogen activator inhibitor type 1 (*SERPINE1*), inducible nitric oxide synthase 2A (*NOS2A*), and VEGF receptor genes (Nagai *et al.* 2005), respectively. *KLF5* is down-regulated with vascular development (Nagai *et al.* 2005), which is in line with the *KLF5* mRNA expression profile observed in the bovine endometrium during the oestrous cycle (increased mRNA levels at days 3.5, 12, 18P4H; cluster 2) and its target TEK (highest mRNA levels at days 18P4L and 0; cluster 5).

In addition to *PLAT, MET, TIMP2* and *MMP2*, the gene products of cystatin M (*CST6*; cluster 1), teratocarcinoma-derived growth factor 1 (*TDGF1*; cluster 1), integrin-β4 (*ITGB4*; cluster 2) and DnaJ-like heat shock protein 40 (*DNAJB4*; cluster 2) are connected with the process invasive growth and are thus potentially involved in the regulation of embryo implantation. Some of these genes were described as negative regulators of invasive growth, e.g. *PLAT* (Lin *et al.* 2001) and *CST6* (Shridhar *et al.* 2004). Expression of integrins in the context of embryo implantation has been investigated in the human and mouse endometrium and in domestic animals (Aplin 1997, Burghardt *et al.* 2002).

A number of genes with higher mRNA levels during the luteal phase are related to the process of cell adhesion. However, only one of these genes – *LGALS3BP*, coding for galectin-3-binding protein, also known as Mac-2-binding protein (cluster 2) – is a candidate for cell–cell adhesion (Inohara *et al.* 1996). In one of our previous studies, *LGALS3BP* mRNA concentration was higher in day 18 pregnant endometrium versus day 18 control endometrium and mRNA expression localised to the luminal epithelium (Bauersachs *et al.* 2006).

During the pre-implantation phase, feeding of the elongating embryo is an important function of the

endometrium. This phase corresponds to the luteal phase of the oestrous cycle. In this context, a number of genes related to several metabolic functions were up-regulated during the luteal phase, such as mitochondrial aconitase 2 (*ACO2*), oxoglutarate dehydrogenase (*OGDH*), isocitrate dehydrogenase 1 (*IDH1*) and *IDH2* (clusters 1, 2, 1, 1), which are involved in the TCA cycle. Furthermore, genes encoding different transporters were identified as up-regulated during the luteal phase. *SLC1A1* (cluster 1) codes for a glutamate transporter that has been detected in the human placenta and suggested to be involved in the active transport of glutamate between the fetal and maternal blood circulation (Noorlander *et al*. 2004). *SLC11A2* (cluster 2) encodes a metal ion transporter expressed in the human placenta (Chong *et al*. 2005). *SLC16A1* (cluster 2) codes for monocarboxylate transporter 8, described as a powerful and specific thyroid hormone (TH) membrane transporter with an important role in the TH transport during fetal development and a specific role in the human placental development (Chan *et al*. 2006). Selenoprotein P (*SEPP1*; cluster 1) is a selenium supply protein (Saito & Takahashi 2002) and ectonucleotide pyrophosphatase/phosphodiesterase 1 (*ENPP1*; cluster 2) regulates extracellular pyrophosphate concentrations (Hessle *et al*. 2002).

The probable functions of the six genes, which were selected for mRNA *in situ* hybridisation, are discussed in the following in detail. These genes were selected based on their potential role for the regulation of the preparation of the endometrium for embryo implantation. In cattle, implantation is non-invasive (placenta epitheliochorealis). The luminal epithelium of the endometrium remains mainly intact except for the formation of giant cells, which are the fusion products of the epithelial and trophoblast cells (Wathes & Wooding 1980). The exchange of nutrients takes place by diffusion through the epithelium, providing an appropriate environment for the developing embryo. Tight junction barriers of the luminal epithelium regulate the passage of ions, water and molecules through the paracellular space (Gonzalez- Mariscal *et al*. 2003) and separate the very different maternal and embryonic milieu (Hees & Sinowatz 1992). Three mRNAs coding for tight junction proteins show differential expression during the oestrous cycle: *CLDN10, CLDN4* and *TJP1* (zona occludens 1). Claudins, for example, are tight junction-associated adhesion molecules involved in the formation of epithelial barriers and are directly involved in intercellular sealing in the simple epithelia (Tsukita & Furuse 2002). The mRNA coding for CLDN10 is strongly up-regulated at oestrus (cluster 5), whereas the mRNA level of CLDN4 is increased at dioestrus with the highest levels during the late luteal phase (cluster 1). *TJP1* mRNA expression showed the lowest levels at day 0, increased until day 12 and decreased thereafter (cluster 2). Both the claudin mRNAs are mainly localised in the luminal epithelium and the superficial glands. *In situ* hybridisation for *TJP1* mRNA showed in addition strong staining of the deeper endometrial glands. In mice, *Tjp1* has been shown to take part in the formation of a temporary barrier in the cells of the primary decidual zone that restricts the passage of injurious stimuli such as maternal immunoglobulins to the embryo (Wang *et al*. 2004). The differential regulation of mRNAs coding for tight junction proteins suggests an important role regarding the preparation of the endometrium for embryo implantation and protection of the embryo from the maternal immune system.

The proprotein convertase subtilisin/kexin type 5 (PCSK5), also known as prohormone convertase 5 or protease PC6, has been shown to convert inactive precursors to their mature active forms (Seidah & Chretien 1999). One of the proteins that first occur as immature precursors and become activated by PCSK5 is VEGF (Popovici *et al*. 2000), which is known to be involved in the regulation of angiogenesis (Carmeliet 2005; see above). Strong induction of PCSK5 protein has been shown in uterine stromal cells specifically at the site of embryo attachment during early pregnancy in mice and during the phase of uterine receptivity and at implantation in primates. *In vivo* blocking of uterine production of PCSK5 protein in mice resulted in total inhibition of implantation (Nie *et al*. 2003, 2005). In contrast, in our present study, the mRNA expression of *PCSK5* was the highest at oestrus and decreased during the luteal phase (cluster 3). *PCSK5* may be an example for species-specific differences of gene expression regulation due to its essential role in the regulation of invasive type of implantation. Furthermore, *in situ* hybridisation revealed a stronger staining in the luminal epithelial cells and the superficial glands than in the stromal cells. The higher mRNA levels observed during oestrus may be associated with angiogenesis and remodelling of the ECM , since PCSK5 has been shown to play a central role in the MT-MMP-MMP-2 proteolytic cascade (Stawowy *et al*. 2005).

MAGED1 mRNA was up-regulated at oestrus (day 0) and down-regulated at dioestrus (cluster 5) and mainly localised in the superficial uterine glands and the luminal epithelium. While the melanoma antigen family genes, *MAGEA* and *MAGEB*, are silent in the normal tissues with the exception of testis and placenta, the *MAGED* genes are expressed ubiquitously. *MAGED1*, also known as *NRAGE*, has been shown to alter the cytoskeleton, to inhibit cell–cell adhesion and to suppress mRNA expression and activity of MMP2 (Chu *et al*. 2007). Another member of this gene family, *MAGED2*, was found as up-regulated during dioestrus (cluster 1), suggesting that genes of this family may also play a role in cellular adhesion processes.

LGALS1 mRNA concentration was increased at oestrus (cluster 5). *In situ* hybridisation revealed pronounced staining in the superficial epithelial glands and weak-to-moderate staining in other cells. The LGALS1 protein, a

multifunctional secreted member of the galectin family, plays a pivotal role in the modulation of cell adhesion, cell growth, inflammation and angiogenesis (Almkvist & Karlsson 2004, von Wolff *et al.* 2005, Thijssen *et al.* 2006). In the human endometrium, LGALS1 protein has been mainly localised in the stromal cells with increased expression in the late secretory phase and in the decidual tissue (von Wolff *et al.* 2005). The contrary spatial and temporal expression of *LGALS1* in the bovine and human endometrium, i.e. between species with different types of placentation, suggests this gene as a further interesting candidate for the regulation of embryo implantation.

In conclusion, the present study revealed distinct dynamic expression profiles and identified numerous genes differentially expressed in the bovine endometrium during the oestrous cycle. The function of many of these genes, especially with respect to the preparation of the endometrium for implantation, is still unknown. The results of this study provide the basis for an in-depth analysis of individual genes/proteins or particularly interesting components of regulatory networks in the bovine uterus during the oestrous cycle and early pregnancy. From a practical perspective, knowledge of physiological changes in the endometrial transcriptome profiles during the oestrous cycle is important for the development of array-based diagnostic procedures to assess aberrant gene expression profiles associated with reduced maternal fertility or metabolic problems of high yielding dairy cows.

Materials and Methods

Pretreatment of animals and collection of endometrial tissue samples

Cyclic heifers (*Bos taurus*, Simmental Fleckvieh) between 17 and 31 months old were synchronised by an i.m. injection of a single dose of 500 μg cloprostenol (Estrumate; Essex Tierarznei, Munich, Germany) at dioestrus. Around 60 h after Estrumate injection, standing heat was identified through observation of sexual behaviour (i.e. toleration, sweating, vaginal mucus). In addition, ultrasound-guided follicle monitoring was performed. Blood samples were taken 2 days before oestrus (day 0), at oestrus and just before slaughtering to determine serum P_4 levels (Prakash *et al.* 1987). For the determination of serum LH levels by a specific RIA (Schams & Karg 1969), blood samples were taken in intervals of 6 hours for the day 0 group. Animals were slaughtered on days 0 (oestrus, within 12 hrs after the LH surge; $n=4$), 3.5 ($n=4$), 12 (dioestrus, luteal phase; $n=4$) and 18 of the oestrus cycle ($n=8$; 4 with high and 4 with low serum P_4 levels) respectively. After slaughter, the uterus was removed, dissected from the surrounding tissue, opened longitudinally and divided into seven sections: corpus plus caudal, middle and cranial parts of the ipsilateral and the contralateral uterine horns (Bauersachs *et al.* 2005). For the analyses in the present study, tissue samples of the middle part of the ipsilateral horn were used. Samples (about 250 mg) were carefully cut from the intercaruncular endometrium with a scalpel within 20 min after slaughter of the donor animal and immediately transferred into vials containing 4 ml RNAlater (Ambion, Huntingdon, Cambridgeshire, UK) and incubated overnight at 4 °C. The samples were stored at −20 °C until further processing. Tissue samples for *in situ* hybridisation were collected from the same animals and the same location within the uterus and fixed by immersion in formalin (3.7%) and then routinely processed for embedding in paraffin. All experiments with animals were conducted with permission from the local veterinary authorities and in accord with the accepted standards of humane animal care.

Preparation of cDNA arrays and cDNA array hybridisation

The generation of the BOE array has been described previously (Bauersachs *et al.* 2007). Fifteen microlitres of the PCR products of the cDNA fragments and 15 μl long oligonucleotides (50 ng/μl; Operon, Cologne, Germany) were transferred to 384-well microtitre plates (Abgene, Epsom, Surrey, UK) containing 15 μl twofold spotting buffer (40 mM Tris–HCl (pH 8.0), 2 M NaCl, 2 mM EDTA, bromophenol blue). A total of 1344 cDNA fragments and 96 oligonucleotides were spotted onto nylon membranes (Nytran Plus; Whatman Schleicher & Schuell, Dassel, Germany) on an area of 20×50 mm using a microarray robot (Omnigrid Accent; Gene Machines, Ann Arbor, MI, USA) and solid pins (SSP015, diameter 0.015 inch; Telechem International, Sunnyvale, CA, USA). Spotting was done six times for each PCR product on the same position for sufficient and equal application. Thirty-two arrays were produced simultaneously. The spotted DNA was denatured by incubation with 0.5 M NaOH for 20 min at room temperature. Subsequently, DNA was immobilised by baking for 30 min at 80 °C and u.v. crosslinking (120 mJ/cm^2; XL-1500 u.v. crosslinker; Spectronics Corp., Westbury, NY, USA). Total RNA was isolated from 20 endometrial tissue samples using TRIzol (Invitrogen), according to the manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry and agarose gel electrophoresis respectively. Double-stranded (ds) cDNA was synthesised starting with 50 μg total RNA using Superscript III (200 U/μl, Invitrogen) and 50 pmol cDNA synthesis primer ($GAGAT_{20}VN$; V=A, C or G) 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 5 μl RNase (0.5 mg/μl, DNase free, from bovine pancreas; Roche Diagnostics) for 90 min at 37 °C. Remaining nucleotides and primers were removed using Microspin S200-HR spin columns (GE Healthcare Life Science, Munich, Germany). Subsequently, cDNA was precipitated with 15 μl of 3 M NaOAc and 150 μl isopropanol. Dry sediment was solubilised in 20 μl of 0.5× Tris/HCl–EDTA (TE) buffer (pH 8.0). The quality of cDNA was determined by agarose gel electrophoresis.

^{33}P-labelled cDNA probes (four for each group) 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-^{33}PdCTP were added to a final volume of 20 μl. Reaction mixtures were incubated for 1 h at

37 °C, heat inactivated for 20 min at 65 °C and purified with ProbeQuant G50 spin columns (GE Healthcare Life Science) to remove unincorporated nucleotides and to estimate labelling efficiency. Hybridisation was done as follows: pre-hybridisation was done for five arrays together in one 15 cm glass hybridisation bottle: 2×10 min 10 ml 0.1× PBS/1% SDS at 85 °C; 3×10 min 10 ml 1× PBS/10% SDS at 65 °C and 1×10 min 10 ml 1× PBS/10% SDS at room temperature. Hybridisation probes were denatured for 15 min at 96 °C immediately before adding them to the hybridisation solution. Hybridisation was done in plastic vials (Poly-Q vials, 18 ml; Beckman Coulter, Munich, Germany) in 2.5 ml 1× PBS (pH 7.5)/10% SDS for 47 h at 65 °C. After hybridisation, the arrays were put together in 15 cm glass hybridisation bottles, five arrays per bottle, and washed as follows: 3×5 min 10 ml 1× PBS/10% SDS at 65 °C; 3×10 min 10 ml 1× PBS/10% SDS at 65 °C; 3×10 min 10 ml 0.1× PBS/1% SDS at 65 °C and 3×5 min 10 ml 1× PBS/1% SDS/2 mM EDTA at 30 °C. Filters were dried by baking at 80 °C for 20 min. The filters were exposed to an imaging plate BAS-SR (Fuji Photo Film Co., purchased from Raytest, Straubenhardt, Germany). Imaging plates were scanned with a phosphor imager (Typhoon Imager; GE Healthcare Life Science). Labelling and hybridisation were done in parallel for all the 20 samples.

Analysis of array data

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession number GSE8337. Array evaluation was done using AIDA Image Analyzer software (version 3.52; Raytest). Background was subtracted with the 'lowest grid dot' function. Raw data were normalised with the BioConductor package vsn (Huber *et al.* 2002). For quality control, normalised data were analysed with a distance matrix and a heatmap based on pair-wise distances (BioConducter package geneplotter). Significance analysis was performed using the 'significance analysis of microarrays' method (SAM, Multiclass; Tusher *et al.* 2001). Data from cDNAs with significant changes during the oestrous cycle was used for cluster analyses with MultiExperiment Viewer 4.0 (Saeed *et al.* 2003). For hierarchical clustering of genes and samples, HCL support trees were used based on mean centred values ($\log_2$ value of a gene minus mean of all 19 $\log_2$ values of this gene). For SOTA (cluster analysis for identification of similar expression profiles), means for each of the five groups were calculated and mean centred (mean $\log_2$ value group minus mean of all the five groups). Quantitatively enriched GO categories were identified using GOTM (Zhang *et al.* 2004) and OntoExpress (Draghici *et al.* 2003). Integrated analysis of different functional databases was done using the 'functional annotation clustering' tool of the DAVID (Dennis *et al.* 2003). Genes were assigned to KEGG pathways (http://www.genome.ad.jp/kegg/tool/search_pathway.html) based on the Entrez Gene ID of the putative human orthologous gene. Interaction networks were built using Pathway Architect software (version 2.0.1; Stratagene). Most of the interactions and cellular localisations provided by the Pathway Architect databases were checked. Additional interactions found in the literature were assigned to the network.

Quantitative real-time RT-PCR

Real-time RT-PCR was performed as described previously (Ulbrich *et al.* 2004). The following primers (PCR product length in parentheses) were used to amplify specific fragments referring to selected mRNAs: *CLDN10* (forward: 5′-CATTTCATGCCAATCAGGG, reverse: 5′-CGCTGGACGGTTACATCC (97 bp)), *IRF1* (forward: 5′-TCTGCTTAATGAACCTGGGG, reverse: 5′-TATCAGGCCAATATAACCCCC (103 bp)), *LGALS1* (forward: 5′-AGTGTCGTGGAGGTATGCATC, reverse: 5′-TGAAGTCACCACCTGCAGAC (133 bp)), *PCKS5* (forward: 5′-ATGTCAAACAGCTGGCGTC, reverse: 5′-TACTTCATGGAGGAGCGGAG (115 bp)), *PLA2 G10* (forward: 5′-TTGTGTGGGAACTACTCACCC, reverse: 5′-TCAAACTGGAAACCAAACTGG (211 bp)), *PFN1* (forward: 5′-CCCCAACCTTCAATATCACG, reverse: 5′-TTTCTTGTTGATCATACCGCC (95 bp)), *SERPING1* (forward: 5′-AAATGACAGCACAGCCAGC, reverse: 5′-CTATCTTCCACTTGGCGCTC (149 bp)), *STC1* (forward: 5′-CATATTCGTCCATGGCCAG, reverse: 5′-GGTTGCCCCAGTAGAGTTAGC (124 bp)), *TDGF1* (forward: 5′-TCATGCAGATTTCATGACCTG, reverse: 5′-TTTAGGGACCACAGGGAAATC (110 bp)), *TNC* (forward: 5′-ACT- TTAGCTCCTGCCAGAYG, reverse: 5′-TAAGTTTCCGGGGACAACTG (124 bp)), *TJP1* (forward: 5′-GCTCTAAAATCATCCGACTCCTC, reverse: 5′-ACAGAAACACAGTTTGCTC- CAAC (104 bp)) and 18S rRNA (forward: 5′-AAGTCTTTGGGTTCCGGG, reverse: 5′-GGACATCTAAGGGCATCACA (365 bp)). All amplified PCR fragments were sequenced to verify the resulting PCR product (MWG-Biotech, Ebersberg, Germany). The annealing temperature (AT) and the appropriate fluorescence acquisition (FA) points for quantification within the fourth step of the amplification segment were as follows: 18S rRNA (AT 60 °C, FA 82 °C); *IRF1*, *LGALS1*, *PLA2G10*, *TNC*, *PFN1* (AT 60 °C, FA 80 °C); *CLDN10* (AT 64 °C, FA 80 °C); *PCKS5* (AT 66 °C, FA 74 °C); *SERPING1* (AT 64 °C, FA 85 °C); *STC1* (AT 66 °C, FA 74 °C); *TDGF1* (AT 60 °C, FA 74 °C) and *TJP1* (AT 64 °C, FA 77 °C). The cycle number required to achieve a definite SYBR Green fluorescence signal (CP) was calculated by the second derivative maximum method (LightCycler software version 4.05; Roche Diagnostics). The CP is correlated inversely with the logarithm of the initial template concentration. The CP determined for the target genes were normalised against 18S rRNA (ΔCP). Differences between the groups of the different cycle stages are expressed by the ΔΔCP as well as the mean fold changes in relation to the cycle stage having the lowest expression (Livak & Schmittgen 2001). The differences between the groups were analysed using one-way ANOVA. The normal distribution was tested by the Kolmogorov–Smirnov method, followed by a Holm–Sidak test to find the significant differences (Sigma-Stat, version 2.03). The overall level of significance (*P* value) is shown in Table 1.

In situ *hybridisation*

In situ hybridisation of 5 µm paraffin sections of the endometrium samples was performed as described previously (Bauersachs *et al.* 2005). The sequences of the antisense oligonucleotides were as follows: *CLDN10*, 5′-ATGGGCAGGAGCCTCACTCT; *CLDN4*, 5′-AACATCGTCACGTCGCAGAC;

LGALS1, 5′-TGTGCCTCCACTTCAACCCT; *MAGED1*, 5′-GTCATCTTCATGAATGGCAAC; *PCSK5*, 5′-GCAGAGCTGTAGTATCAGCTA; *TJP1*, 5′-AGAGGCCTCACATGAAGCCA. Negative controls were done by omitting the oligonucleotide probe and by hybridisation with sense oligonucleotide probes (complementary sequence of the antisense oligonucleotides).

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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References

Abe K, Tilan JU & Zukowska Z 2007 NPY and NPY receptors in vascular remodeling. *Current Topics in Medicinal Chemistry* **7** 1704–1709.

Ace CI & Okulicz WC 2004 Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. *Reproductive Biology and Endocrinology* **2** 54.

Almkvist J & Karlsson A 2004 Galectins as inflammatory mediators. *Glycoconjugate Journal* **19** 575–581.

Aplin JD 1997 Adhesion molecules in implantation. *Reviews of Reproduction* **2** 84–93.

Atkinson BA, King GJ & Amoroso EC 1984 Development of the caruncular and intercaruncular regions in the bovine endometrium. *Biology of Reproduction* **30** 763–774.

Bauersachs S, Blum H, Mallok S, Wenigerkind H, Rief S, Prelle K & Wolf E 2003 Regulation of ipsilateral and contralateral bovine oviduct epithelial cell function in the postovulation period: a transcriptomics approach. *Biology of Reproduction* **68** 1170–1177.

Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prelle K, Wenigerkind H, Einspanier R, Blum H & Wolf E 2004 Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *Journal of Molecular Endocrinology* **32** 449–466.

Bauersachs S, Ulbrich SE, Gross K, Schmidt SE, Meyer HH, Einspanier R, Wenigerkind H, Vermehren M, Blum H, Sinowatz F *et al.* 2005 Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *Journal of Molecular Endocrinology* **34** 889–908.

Bauersachs S, Ulbrich SE, Gross K, Schmidt SEM, Meyer HH, Wenigerkind H, Vermehren M, Sinowatz F, Blum H, Wolf E *et al.* 2006 Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction* **132** 319–331.

Bauersachs S, Mitko K, Blum H & Wolf E 2007 Technical note: Bovine oviduct and endometrium array version 1: a tailored tool for studying bovine endometrium biology and pathophysiology. *Journal of Dairy Science* **90** 4420–4423.

Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE & Bazer FW 2002 Integrins and extracellular matrix proteins at the maternal–fetal interface in domestic animals. *Cells, Tissues, Organs* **172** 202–217.

Carmeliet P 2005 VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69** (Supplement 3) 4–10.

Chan SY, Franklyn JA, Pemberton HN, Bulmer JN, Visser TJ, McCabe CJ & Kilby MD 2006 Monocarboxylate transporter 8 expression in the human placenta: the effects of severe intrauterine growth restriction. *Journal of Endocrinology* **189** 465–471.

Chong WS, Kwan PC, Chan LY, Chiu PY, Cheung TK & Lau TK 2005 Expression of divalent metal transporter 1 (DMT1) isoforms in first trimester human placenta and embryonic tissues. *Human Reproduction* **20** 3532–3538.

Chu CS, Xue B, Tu C, Feng ZH, Shi YH, Miao Y & Wen CJ 2007 NRAGE suppresses metastasis of melanoma and pancreatic cancer *in vitro* and *in vivo*. *Cancer Letters* **250** 268–275.

Dennis G Jr, Sherman BT, Hosack DA, Yang J, Gao W, Lane HC & Lempicki RA 2003 DAVID: database for annotation, visualization, and integrated discovery. *Genome Biology* **4** P3.

Diaz VM, Planaguma J, Thomson TM, Reventos J & Paciucci R 2002 Tissue plasminogen activator is required for the growth, invasion, and angiogenesis of pancreatic tumor cells. *Gastroenterology* **122** 806–819.

Draghici S, Khatri P, Martins RP, Ostermeier GC & Krawetz SA 2003 Global functional profiling of gene expression. *Genomics* **81** 98–104.

Fridman R, Fuerst TR *et al.* 1992 Domain structure of human 72-kDa gelatinase/type IV collagenase. Characterization of proteolytic activity and identification of the tissue inhibitor of metalloproteinase-2 (TIMP-2) binding regions. *Journal of Biological Chemistry* **267** 15398–15405.

Gipson IK, Blalock T, Tisdale A, Spurr-Michaud S, Allcorn S, Stavreus-Evers A & Gemzell K 2007 MUC16 is lost from the uterodome (pinopode) surface of the receptive human endometrium: *in vitro* evidence that MUC16 is a barrier to trophoblast adherence. *Biology of Reproduction* **78** 134–142.

Goff AK 2004 Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. *Biology of Reproduction* **71** 11–16.

Gonzalez-Mariscal L, Betanzos A, Nava P & Jaramillo BE 2003 Tight junction proteins. *Progress in Biophysics and Molecular Biology* **81** 1–44.

Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW & Spencer TE 2006 Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. *Biology of Reproduction* **74** 383–394.

Greiss FC Jr & Anderson SG 1969 Uterine vescular changes during the ovarian cycle. *American Journal of Obstetrics and Gynecology* **103** 629–640.

Hees H & Sinowatz F 1992 *Histologie – Kurzlehrbuch der Zytologie und mikroskopischen Anatomie*. Deutscher Ärzte-Verlag GmbH, Cologne: Cologne.

Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R & Millan JL 2002 Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *PNAS* **99** 9445–9449.

Horcajadas JA, Pellicer A & Simon C 2007 Wide genomic analysis of human endometrial receptivity: new times, new opportunities. *Human Reproduction Update* **13** 77–86.

Huber W, von Heydebreck A, Sultmann H, Poustka A & Vingron M 2002 Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* **18** (Supplement 1) S96–S104.

Inohara H, Akahani S, Koths K & Raz A 1996 Interactions between galectin-3 and Mac-2-binding protein mediate cell–cell adhesion. *Cancer Research* **56** 4530–4534.

Kaeoket K, Persson E & Dalin AM 2001 The sow endometrium at different stages of the oestrous cycle: studies on morphological changes and infiltration by cells of the immune system. *Animal Reproduction Science* **65** 95–114.

Kaushal GP, Walker PD & Shah SV 1994 An old enzyme with a new function: purification and characterization of a distinct matrix-degrading metalloproteinase in rat kidney cortex and its identification as meprin. *Journal of Cell Biology* **126** 1319–1327.

Klein C, Bauersachs S *et al.* 2006 Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. *Biology of Reproduction* **74** 253–264.

Li XH, Kakkad B & Ong DE 2004 Estrogen directly induces expression of retinoic acid biosynthetic enzymes, compartmentalized between the epithelium and underlying stromal cells in rat uterus. *Endocrinology* **145** 4756–4762.

Lin VC, Eng AS, Hen NE, Ng EH & Chowdhury SH 2001 Effect of progesterone on the invasive properties and tumor growth of progesterone receptor-transfected breast cancer cells MDA-MB-231. *Clinical Cancer Research* **7** 2880–2886.

Livak KJ & Schmittgen TD 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta *C*(T)) method. *Methods* **25** 402–408.

Ma PC, Maulik G, Christensen J & Salgia R 2003 c-Met: structure, functions and potential for therapeutic inhibition. *Cancer Metastasis Reviews* **22** 309–325.

Morisada T, Kubota Y, Urano T, Suda T & Oike Y 2006 Angiopoietins and angiopoietin-like proteins in angiogenesis. *Endothelium* **13** 71–79.

Murwantoko , Yano M, Ueta Y, Murasaki A, Kanda H, Oka C & Kawaichi M 2004 Binding of proteins to the PDZ domain regulates proteolytic activity of HtrA1 serine protease. *Biochemical Journal* **381** 895–904.

Nagai R, Suzuki T, Aizawa K, Shindo T & Manabe I 2005 Significance of the transcription factor KLF5 in cardiovascular remodeling. *Journal of Thrombosis and Haemostasis* **3** 1569–1576.

Nagata K 1996 Hsp47: a collagen-specific molecular chaperone. *Trends in Biochemical Sciences* **21** 22–26.

Nie GY, Li Y, Minoura H, Findlay JK & Salamonsen LA 2003 Specific and transient up-regulation of proprotein convertase 6 at the site of embryo implantation and identification of a unique transcript in mouse uterus during early pregnancy. *Biology of Reproduction* **68** 439–447.

Nie G, Li Y, Wang M, Liu YX, Findlay JK & Salamonsen LA 2005 Inhibiting uterine PC6 blocks embryo implantation: an obligatory role for a proprotein convertase in fertility. *Biology of Reproduction* **72** 1029–1036.

Nie G, Hale K, Li Y, Manuelpillai U, Wallace EM & Salamonsen LA 2006 Distinct expression and localization of serine protease HtrA1 in human endometrium and first-trimester placenta. *Developmental Dynamics* **235** 3448–3455.

Noorlander CW, de Graan PN, Nikkels PG, Schrama LH & Visser GH 2004 Distribution of glutamate transporters in the human placenta. *Placenta* **25** 489–495.

Oka C, Tsujimoto R *et al.* 2004 HtrA1 serine protease inhibits signaling mediated by TGFβ family proteins. *Development* **131** 1041–1053.

Pak DT & Sheng M 2003 Targeted protein degradation and synapse remodeling by an inducible protein kinase. *Science* **302** 1368–1373.

Ponnampalam AP, Weston GC, Trajstman AC, Susil B & Rogers PA 2004 Molecular classification of human endometrial cycle stages by transcriptional profiling. *Molecular Human Reproduction* **10** 879–893.

Popovici RM, Kao LC & Giudice LC 2000 Discovery of new inducible genes in *in vitro* decidualized human endometrial stromal cells using microarray technology. *Endocrinology* **141** 3510–3513.

Prakash BS, Meyer HH, Schallenberger E & van de Wiel DF 1987 Development of a sensitive enzymeimmunoassay (EIA) for progesterone determination in unextracted bovine plasma using the second antibody technique. *Journal of Steroid Biochemistry* **28** 623–627.

Roberts RM & Bazer FW 1988 The functions of uterine secretions. *Journal of Reproduction and Fertility* **82** 875–892.

Rolli-Derkinderen M, Machavoine F, Baraban JM, Grolleau A, Beretta L & Dy M 2003 ERK and p38 inhibit the expression of 4E-BP1 repressor of translation through induction of Egr-1. *Journal of Biological Chemistry* **278** 18859–18867.

Ruesse I & Sinowatz F 1998 Weiblicher Sexualzyklus. In *Lehrbuch der Embryologie der Haustiere*, edn 2, pp 93–116. Berlin: Parey Buchvelag.

Saeed AI, Sharov V *et al.* 2003 TM4: a free, open-source system for microarray data management and analysis. *BioTechniques* **34** 374–378.

Saito Y & Takahashi K 2002 Characterization of selenoprotein P as a selenium supply protein. *European Journal of Biochemistry* **269** 5746–5751.

Schams D & Karg H 1969 Radioimmunologic determination of LH in bovine serum, with special attention to the estrous cycle. *Acta Endocrinologica* **61** 96–103.

Seidah NG & Chretien M 1999 Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. *Brain Research* **848** 45–62.

Shridhar R, Zhang J, Song J, Booth BA, Kevil CG, Sotiropoulou G, Sloane BF & Keppler D 2004 Cystatin M suppresses the malignant phenotype of human MDA-MB-435S cells. *Oncogene* **23** 2206–2215.

Spencer TE, Burghardt RC, Johnson GA & Bazer FW 2004*a* Conceptus signals for establishment and maintenance of pregnancy. *Animal Reproduction Science* **82–83** 537–550.

Spencer TE, Johnson GA, Burghardt RC & Bazer FW 2004*b* Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biology of Reproduction* **71** 2–10.

Stawowy P, Meyborg H *et al.* 2005 Furin-like proprotein convertases are central regulators of the membrane type matrix metalloproteinase-pro-matrix metalloproteinase-2 proteolytic cascade in atherosclerosis. *Circulation* **111** 2820–2827.

Takahashi T, Eitzman B, Bossert NL, Walmer D, Sparrow K, Flanders KC, McLachlan J & Nelson KG 1994 Transforming growth factors beta 1, beta 2, and beta 3 messenger RNA and protein expression in mouse uterus and vagina during estrogen-induced growth: a comparison to other estrogen-regulated genes. *Cell Growth and Differentiation* **5** 919–935.

Takeda N, Maemura K, Imai Y, Harada T, Kawanami D, Nojiri T, Manabe I & Nagai R 2004 Endothelial PAS domain protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, Flt-1. *Circulation Research* **95** 146–153.

Tan YF, Li FX, Piao YS, Sun XY & Wang YL 2003 Global gene profiling analysis of mouse uterus during the oestrous cycle. *Reproduction* **126** 171–182.

Thijssen VL, Postel R, Brandwijk RJ, Dings RP, Nesmelova I, Satijn S, Vethofstad N, Nakabeppu Y, Baum LG, Bakkers J *et al.* 2006 Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *PNAS* **103** 15975–15980.

Tsukita S & Furuse M 2002 Claudin-based barrier in simple and stratified cellular sheets. *Current Opinion in Cell Biology* **14** 531–536.

Tusher VG, Tibshirani R & Chu G 2001 Significance analysis of microarrays applied to the ionizing radiation response. *PNAS* **98** 5116–5121.

Ulbrich SE, Schoenfelder M, Thoene S & Einspanier R 2004 Hyaluronan in the bovine oviduct–modulation of synthases and receptors during the extrous cycle. *Molecular and Cellular Endoctinology* **214** 9–18.

Wang X, Matsumoto H, Zhao X, Das SK & Paria BC 2004 Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation. *Journal of Cell Science* **117** 53–62.

Wathes DC & Wooding FB 1980 An electron microscopic study of implantation in the cow. *American Journal of Anatomy* **159** 285–306.

von Wolff M, Wang X, Gabius HJ & Strowitzki T 2005 Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. *Molecular Human Reproduction* **11** 189–194.

Yamada O, Todoroki J, Takahashi T & Hashizume K 2002 The dynamic expression of extracellular matrix in the bovine endometrium at implantation. *Journal of Veterinary Medical Science* **64** 207–214.

Zhang B, Schmoyer D, Kirov S & Snoddy J 2004 GO tree machine (GOTM): a web-based platform for interpreting sets of interesting genes using gene ontology hierarchies. *BMC Bioinformatics* **5** 16.

Zheng J, Bird IM, Chen DB & Magness RR 2005 Angiotensin II regulation of ovine fetoplacental artery endothelial functions: interactions with nitric oxide. *Journal of Physiology* **565** 59–69.

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6 Perspectives

Based on the results of this work it can be concluded that the BOE array is applicable for the analysis of mRNA expression profiles in bovine endometrial samples. There are manifold applications for the BOE array in basic and applied research for the analysis of normal and pathological physiology associated with endometrial transcriptome changes. One interesting field for the use of the BOE array are the influences of assisted reproduction techniques (ART) on endometrial transcriptome profiles. ART become increasingly important in human reproductive medicine and in animal breeding and biotechnology as well. Although in vitro fertilisation and intracytoplasmic sperm injection have been established to the level of clinical application, there are recent reports of a higher frequency of epigenetic abnormalities in offspring derived from ART as compared to natural reproduction (Horsthemke and Ludwig 2005). Somatic cell nuclear transfer cloning, which has been successful in a number of species, is particularly critical with respect to epigenetic abnormalities of the resulting embryos, foetuses and offspring (reviewed in (Shi, Zakhartchenko *et al.* 2003)). However, it is largely unclear whether and how epigenetic abnormalities cause developmental abnormalities and abortions of cloned embryos or foetuses. A number of studies revealed placental abnormalities as primary cause of pregnancy loss after transfer of bovine SCNT embryos. Placental changes include a reduced number, but increased size of placentomes (Chavatte-Palmer, Heyman *et al.* 2002; Constant, Guillomot *et al.* 2006). Therefore, it would be interesting to study, how and when these changes of placental functionality are induced, which could be examined by evaluating transcriptome profiles of endometrium samples in response to SCNT embryos vs. embryos derived by in vitro fertilisation.

Furthermore, future applications of the BOE array could be systematic studies of interrelations between the metabolic status of dairy cows and the functionality of the endometrium to identify differentially expressed genes or characteristic expression profiles of sets of genes that could be used for differential diagnosis of fertility problems. Currently it is discussed, whether the decrease in reproductive performance of high performance milk cows (Butler 1998) is directly due to the high milk yield, negative energy balance and associated metabolic problems (“the physiological hypothesis”) or if there has been an unintended selection for gene variants with a negative effect on fertility during the efficient selection for high milk yield (“the genetic hypothesis”). In theory, beneath malfunctions of the ovaries, problems related to uterus physiology (e.g. disturbed uterine receptivity leading to early embryonic mortality) may be responsible for the overall alarming fertility problems in high yielding dairy cows. It has been shown that stimulation of milk production by increased intake of dietary protein leads to an increase in blood urea nitrogen (Ishikawa, Kaisho *et al.*) (Ishikawa, Kaisho *et al.*) and is often associated with decreased fertility (Butler 1998). Early studies of dairy cows involving embryo collection and evaluation did not provide evidence that high dietary protein has an impact on ovarian follicular development, ovulation, or

fertilisation of oocytes (Blanchard, Ferguson *et al.* 1990; Garcia-Bojalil, Staples *et al.* 1994). Thus, disturbances of early embryonic development and embryo-maternal interactions are more likely an explanation for fertility problems associated with high yield and protein feeding. Although it is not clear whether the embryonic or the maternal compartment is more critical, there is evidence that high protein diet and the associated increase in BUN may change the uterine milieu, e.g. the uterine pH, which may have important implications for the transcriptional activity and functions of the endometrium. Interestingly, bovine endometrial cells in culture are known to respond directly to increasing urea concentrations with increased secretion of $PGF_{2\alpha}$ (Butler 1998) which induces luteolysis in vivo (Wolf, Arnold *et al.* 2003). Moreover, there is evidence for embryotoxic effects of $PGF_{2\alpha}$ in the pre-implantation period (Buford, Ahmad *et al.* 1996) . These observations provide a plausible link between elevated plasma urea nitrogen concentrations and decreased fertility. However, the molecular mechanisms underlying the effects of increased BUN or other metabolic changes on endometrial functions are incompletely understood and need to be studied in vivo. The use of transcriptome profiling of endometrial biopsies from metabolically well-characterised cows in different situations of milk yield and energy balance could help to further characterise these interrelations. Such studies could clarify metabolic influences on the functionality of the endometrium and eventually make an important contribution to the fertility monitoring in high-yielding cows.

In addition to these questions related to basic research, endometrial transcriptome profiles may have important applications for the cattle breeding industry. Future studies will clarify whether transcriptome profiles of endometrium biopsies taken at a specific stage of the oestrous cycle are indicative of the fertility status. If this turns out to be the case, detailed molecular phenotyping could be combined with genotyping using a high-density marker set. Thus associations between a favourable endometrial transcriptome profile and specific genetic markers, could be established, eventually realising the concept of genomic selection (Meuwissen, Hayes *et al.* 2001) for fertility.

For the further development of the BOE array establishing a non-radioactive microarray technology in addition to our approved technique, which is based on radioactive labelleing, would be important to permit robust serial analyses. The first step could be for instance to establish a microarray technology based on long oligonucleotides as probes and detection via fluorescence labelling. This technical improvement would permit the analysis of large sets of samples at moderate costs and the automation of hybridisation and washing of microarrays, which is not possible with radioactivity-based techniques.

Future RNA expression studies should also take non-coding RNAs and microRNAs, important regulators of translation, into account. In addition to profiling at the RNA level proteomics studies of endometrium and of the uterine fluid will be essential to identify biologically relevant protein candidates. Novel proteomics techniques, such as highly

sensitive saturation labelling (Kondo and Hirohashi 2006), will further increase the analytical depth of proteome studies and facilitate analyses of microdissected tissues.

7 Summary

Dynamic transcriptome profiling of bovine endometrium during the oestrous cycle

Fertility problems are the main reason for slaughter of high-performance milk cows, because prolonged calving intervals result in financial losses for the farmer and retard genetic progress. Genetic improvement of fertility would be of great benefit, but functional traits for effective selection are missing. Recent advances in functional genomics tools like DNA microarrays could be the key to identify gene expression patterns in the endometrium that correlate with maternal fertility. Therefore, a bovine oviduct and endometrium cDNA array was established that contains a set of 1,440 cDNA clones and long oligonucleotides representing 950 different genes. The major part of these genes results from a series of differential gene expression studies in endometrium (different stages of the oestrous cycle, day 18 and day 15 pregnant vs. nonpregnant) and oviduct epithelial cells (different stages of the oestrous cycle).

Using this custom-made cDNA array the response of the endometrium was studied to the changing hormonal environment during the bovine oestrous cycle. Endometrium samples were recovered from Simmental heifers slaughtered on day 0 (oestrus), 3.5 (metoestrus), 12 (dioestrus) and 18. The latter group was divided into animals with high (late dioestrus) and low progesterone levels (preoestrus). Statistical analysis with the Significance Analysis of Microarrays (SAM) method revealed 269 genes exhibiting significant changes in their transcript levels during the oestrous cycle in distinct temporal patterns. Two major types of expression profiles were observed, which showed the highest mRNA levels during the oestrus phase or the highest levels during the luteal phase, respectively. A minor group of genes exhibited the highest mRNA levels on day 3.5. Gene ontology (GO) analyses revealed GO categories related to extracellular matrix remodelling, transport, and cell growth and morphogenesis enriched at oestrus, whereas immune response and particular metabolic pathways were overrepresented at dioestrus.

Generation of gene interaction networks uncovered genes possibly involved in biological processes important for establishment of early pregnancy, such as endometrial remodelling (e.g. collagen genes, *MMP2*, *TIMP1*), regulation of angiogenesis (e.g. *ANGPTL2*, *TEK*), regulation of invasive growth (e.g. *PCSK5*, tight junction proteins, *ITGB4*), cell adhesion (e.g. *MUC16*, *LGALS3BP*) and embryo feeding (e.g. *SLC1A1*, *ENPP1*). Localisation of mRNA expression in the endometrium was analysed for *CLDN4*, *CLDN10*, *TJP1*, *PCSK5*, *MAGED1*, and *LGALS1*.

Future application of the BOE array, based on the knowledge from the cycle study, could be the use in systematic studies of interactions between the metabolic status and functionality of the endometrium to identify genes that could be used for differential diagnosis of fertility problems.

8 Zusammenfassung

Dynamische Transkriptionsprofile während des Sexualzyklus im Endometrium beim Rind

Fruchtbarkeitsprobleme sind die Hauptursache für die Schlachtung von Hochleistungs-Milchkühen, da verlängerte Zwischenkalbezeiten finanzielle Verluste für den Tierhalter und eine Verzögerung des Züchtungsfortschrittes bedeuten. Eine positive züchterische Beeinflussung der Fruchtbarkeit wäre von großem Vorteil, ist aber derzeit nicht möglich, da Funktionsparameter für eine effektive Selektion fehlen. Die neuesten Fortschritte im Bereich der funktionalen Genomanalyse, wie z.B. DNA-Microarrays könnten der Schlüssel zur Identifizierung von Genexpressionsmustern im Endometrium, die mit der maternalen Fertilität korrelieren, sein. Daher wurde ein bovines Ovidukt- und Endometrium-cDNA-Array entwickelt, welches ein Set von 1.400 cDNA-Klonen und langen Oligonukleotiden enthält, die 950 unterschiedliche Gene repräsentieren. Der Großteil dieser Gene stammt aus einer Folge von verschiedenen Genexpressionsstudien im Endometrium (verschiedene Zyklusstadien, Tag 18 und Tag 15 trächtig vs. nichtträchtige Tiere) und in Eileiterepithelzellen (verschiedene Stadien des Sexualzyklus).

Mit Hilfe dieses selbst hergestellten cDNA-Arrays wurde die Reaktion des Endometriums auf die sich verändernden Hormonspiegel während des bovinen Sexualzyklus auf der Ebene der mRNA untersucht. Endometriumproben wurden von Färsen der Rasse Simmentaler Fleckvieh gewonnen, die am Zyklustag 0 (Östrus), 3,5 (Metöstrus), 12 (Diöstrus) und 18 geschlachtet wurden. Die letzte Gruppe wurde unterteilt in Tiere mit hohem (später Diöstrus) oder niedrigen Blutprogesteronwerten (Proöstrus) zum Schlachtzeitpunkt. Statistische Analysen mit der SAM-Methode (Significance Analysis of Microarrays) ergaben 269 Gene, deren mRNA-Konzentrationen sich während des Zyklus in ausgeprägten zeitlichen Mustern veränderten. Zwei Haupttypen von Expressionsmustern wurden beobachtet, die ihre höchsten mRNA-Spiegel entweder während des Östrus oder der Lutealphase haben. Eine kleinere Gruppe von Genen zeigte die höchsten mRNA-Spiegel an Tag 3,5. Gene Ontology (GO) Analysen ergaben während des Östrus angereicherte GO-Kategorien, die mit dem Umbau der extrazellulären Matrix, Transportprozessen, Zellwachstum und –umwandlung in Zusammenhang stehen, während im Diöstrus Kategorien wie Immunantwort und bestimmte Stoffwechsel-Pathways überrepräsentiert waren.

Mit Hilfe der Erstellung von Interaktionsnetzwerken wurden Gene identifiziert, die in biologische Prozesse involviert sind, welche für die Aufrechterhaltung der frühen Trächtigkeit wichtig sein könnten. Zu diesen Prozessen gehören der Umbau des Endometriums (z.B. Kollagene, *MMP2*, *TIMP1*), die Regulierung der Angiogenese (z.B. *ANGPTL2*, *TEK*), die Regulierung von invasiven Wachstumspozessen (z.B. *PCSK5*, Tight junction Proteine, *ITGB4*) und die Ernährung des Embryos (z.B. *SLC1A1*, *ENPP1*). Zur weiteren

Charakterisierung ausgewählter Gene wurde die Lokalisation der mRNA Expression im Endometrium für *CLDN4*, *CLDN10*, *TJP1*, *PCSK5*, *MAGED1* und *LGALS1* untersucht.

Basierend auf den Ergebnissen der dynamischen Expressionsstudie während des Zyklus könnten ein zukünftiges Anwendungsgebiet des BOE-Arrays systematischen Studien von Interaktionen zwischen dem metabolischen Status und der Funktionalität des Endometriums sein, um Gene zu finden, die zur Differentialdiagnostik bei Fruchtbarkeitsproblemen genutzt werden können.

9 References

Ace CI, Okulicz WC (2004) Microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase. *Reprod.Biol.Endocrinol.* **2**, 54.

Andrade PM, Silva ID, Borra RC, de Lima GR, Baracat EC (2002) Estrogen regulation of uterine genes in vivo detected by complementary DNA array. *Horm.Metab Res.* **34**, 238-244.

Bauersachs S, Rehfeld S, Ulbrich SE, Mallok S, Prelle K, Wenigerkind H, Einspanier R, Blum H, Wolf E (2004) Monitoring gene expression changes in bovine oviduct epithelial cells during the oestrous cycle. *J.Mol.Endocrinol.* **32**, 449-466.

Bauersachs S, Ulbrich SE*, et al.* (2005) Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. *J.Mol.Endocrinol.* **34**, 889-908.

Bauersachs S, Ulbrich SE*, et al.* (2006) Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction* **132**, 319-31.

Blanchard T, Ferguson J, Love L, Takeda T, Henderson B, Hasler J, Chalupa W (1990) Effect of dietary crude-protein type on fertilization and embryo quality in dairy cattle. *Am J Vet Res* **51**, 905-8.

Borthwick JM, Charnock-Jones DS, Tom BD, Hull ML, Teirney R, Phillips SC, Smith SK (2003) Determination of the transcript profile of human endometrium. *Mol.Hum.Reprod.* **9**, 19-33.

Buford WI, Ahmad N, Schrick FN, Butcher RL, Lewis PE, Inskeep EK (1996) Embryotoxicity of a regressing corpus luteum in beef cows supplemented with progestogen. *Biol Reprod* **54**, 531-7.

Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW (2002) Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues.Organs* **172**, 202-217.

Butler WR (1998) Review: effect of protein nutrition on ovarian and uterine physiology in dairy cattle. *J Dairy Sci* **81**, 2533-9.

Canales RD, Luo Y*, et al.* (2006) Evaluation of DNA microarray results with quantitative gene expression platforms. *Nat Biotechnol* **24**, 1115-22.

Chavatte-Palmer P, Heyman Y, Richard C, Monget P, LeBourhis D, Kann G, Chilliard Y, Vignon X, Renard JP (2002) Clinical, hormonal, and hematologic characteristics of bovine calves derived from nuclei from somatic cells. *Biol Reprod* **66**, 1596-603.

Constant F, Guillomot M, Heyman Y, Vignon X, Laigre P, Servely JL, Renard JP, Chavatte-Palmer P (2006) Large offspring or large placenta syndrome? Morphometric analysis of late gestation bovine placentomes from somatic nuclear transfer pregnancies complicated by hydrallantois. *Biol Reprod* **75**, 122-30.

Deng L, Shipley GL, Loose-Mitchell DS, Stancel GM, Broaddus R, Pickar JH, Davies PJ (2003) Coordinate regulation of the production and signaling of retinoic acid by estrogen in the human endometrium. *J.Clin.Endocrinol.Metab* **88**, 2157-2163.

Duffy P, Crowe MA, Boland MP, Roche JF (2000) Effect of exogenous LH pulses on the fate of the first dominant follicle in postpartum beef cows nursing calves. *J Reprod Fertil* **118**, 9-17.

Garcia-Bojalil CM, Staples CR, Thatcher WW, Drost M (1994) Protein intake and development of ovarian follicles and embryos of superovulated nonlactating dairy cows. *J Dairy Sci* **77**, 2537-48.

Garrett JE, Geisert RD, Zavy MT, Morgan GL (1988) Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J Reprod Fertil* **84**, 437-46.

Godkin JD, Dore JJ (1998) Transforming growth factor beta and the endometrium. *Rev Reprod* **3**, 1-6.

Goff AK (2004) Steroid hormone modulation of prostaglandin secretion in the ruminant endometrium during the estrous cycle. *Biol.Reprod.* **71**, 11-16.

Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE (2006) Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. *Biol Reprod* **74**, 383-94.

Gredler B (2006) Neue Ansätze zur Zuchtwertschätzung Fruchtbarkeit. *DGFZ-Schriftenreihe* 68-76.

Hewitt SC, Deroo BJ, Hansen K, Collins J, Grissom S, Afshari CA, Korach KS (2003) Estrogen receptor-dependent genomic responses in the uterus mirror the biphasic physiological response to estrogen. *Mol Endocrinol* **17**, 2070-83.

Horsthemke B, Ludwig M (2005) Assisted reproduction: the epigenetic perspective. *Hum Reprod Update* **11**, 473-82.

Humblot P (2001) Use of pregnancy specific proteins and progesterone assays to monitor pregnancy and determine the timing, frequencies and sources of embryonic mortality in ruminants. *Theriogenology* **56**, 1417-1433.

Ishikawa J, Kaisho T*, et al.* (1995) Molecular cloning and chromosomal mapping of a bone marrow stromal cell surface gene, BST2, that may be involved in pre-B-cell growth. *Genomics* **26**, 527-534.

Jackson V, Chalkley R (1974) The binding of estradiol-17 beta to the bovine endometrial nuclear membrane. *J Biol Chem* **249**, 1615-26.

Kao LC, Tulac S*, et al.* (2002) Global gene profiling in human endometrium during the window of implantation. *Endocrinology* **143**, 2119-2138.

Kesner JS, Padmanabhan V, Convey EM (1982) Estradiol induces and progesterone inhibits the preovulatory surges of luteinizing hormone and follicle-stimulating hormone in heifers. *Biol Reprod* **26**, 571-8.

Klein C, Bauersachs S*, et al.* (2006) Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. *Biol Reprod* **74**, 253-64.

Kondo T, Hirohashi S (2006) Application of highly sensitive fluorescent dyes (CyDye DIGE Fluor saturation dyes) to laser microdissection and two-dimensional difference gel electrophoresis (2D-DIGE) for cancer proteomics. *Nat Protoc* **1**, 2940-56.

Larson SF, Butler WR, Currie WB (1997) Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *J Dairy Sci* **80**, 1288-95.

Mann GE, Lamming GE (2001) Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* **121**, 175-80.

Margulies M, Egholm M*, et al.* (2005) Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**, 376-80.

Martin J, Dominguez F, Avila S, Castrillo JL, Remohi J, Pellicer A, Simon C (2002) Human endometrial receptivity: gene regulation. *J.Reprod.Immunol.* **55**, 131-139.

Meuwissen TH, Hayes BJ, Goddard ME (2001) Prediction of total genetic value using genome-wide dense marker maps. *Genetics* **157**, 1819-29.

Nephew KP, McClure KE, Ott TL, Dubois DH, Bazer FW, Pope WF (1991) Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. *Biol Reprod* **44**, 536-9.

Nickel R, Schummer A, Seiferle E (1999) Lehrbuch der Anatomie der Haustiere, Band II Eingeweide, 8., vollständig neubearbeitete Auflage, Parey Buchverlag Berlin.

Patterson TA, Lobenhofer EK*, et al.* (2006) Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. *Nat Biotechnol* **24**, 1140-50.

Ponnampalam AP, Weston GC, Trajstman AC, Susil B, Rogers PA (2004) Molecular classification of human endometrial cycle stages by transcriptional profiling. *Mol Hum Reprod* **10**, 879-93.

Reinartz J, Bruyns E, Lin JZ, Burcham T, Brenner S, Bowen B, Kramer M, Woychik R (2002) Massively parallel signature sequencing (MPSS) as a tool for in-depth quantitative gene expression profiling in all organisms. *Brief Funct Genomic Proteomic* **1**, 95-104.

Riesewijk A, Martin J, van Os R, Horcajadas JA, Polman J, Pellicer A, Mosselman S, Simon C (2003) Gene expression profiling of human endometrial receptivity on days LH+2 versus LH+7 by microarray technology. *Mol.Hum.Reprod.* **9**, 253-264.

Roberts RM, Bazer FW (1988) The functions of uterine secretions. *J.Reprod.Fertil.* **82**, 875-892.

Ruesse I, Sinowatz F (1998) 'Lehrbuch der Embryologie der Haustiere.' (Parey Buchverlag: Berlin)

Shaffer C (2007) Next-generation sequencing outpaces expectations. *Nat Biotechnol* **25**, 149.

Shi L, Reid LH*, et al.* (2006) The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* **24**, 1151-61.

Shi W, Zakhartchenko V, Wolf E (2003) Epigenetic reprogramming in mammalian nuclear transfer. *Differentiation* **71**, 91-113.

Shippy R, Fulmer-Smentek S*, et al.* (2006) Using RNA sample titrations to assess microarray platform performance and normalization techniques. *Nat Biotechnol* **24**, 1123-31.

Silvia WJ, Lewis GS, McCracken JA, Thatcher WW, Wilson L, Jr. (1991) Hormonal regulation of uterine secretion of prostaglandin F2 alpha during luteolysis in ruminants. *Biol Reprod* **45**, 655-63.

Spencer TE, Bazer FW (2002) Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci.* **7**, d1879-d1898.

Spencer TE, Johnson GA, Burghardt RC, Bazer FW (2004) Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol.Reprod.* **71**, 2-10.

Starbuck GR, Darwash AO, Lamming GE (1997) The importance of progesterone during early pregnancy in the dairy cow. *Cattle Practice*, 397-399.

Talbi S, Hamilton AE*, et al.* (2006) Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. *Endocrinology* **147**, 1097-121.

Tan YF, Li FX, Piao YS, Sun XY, Wang YL (2003) Global gene profiling analysis of mouse uterus during the oestrous cycle. *Reproduction.* **126**, 171-182.

Tanaka TS, Jaradat SA*, et al.* (2000) Genome-wide expression profiling of mid-gestation placenta and embryo using a 15,000 mouse developmental cDNA microarray. *Proc.Natl.Acad.Sci.U.S.A* **97**, 9127-9132.

Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL (1988) Association between energy balance and luteal function in lactating dairy cows. *J Dairy Sci* **71**, 1063-72.

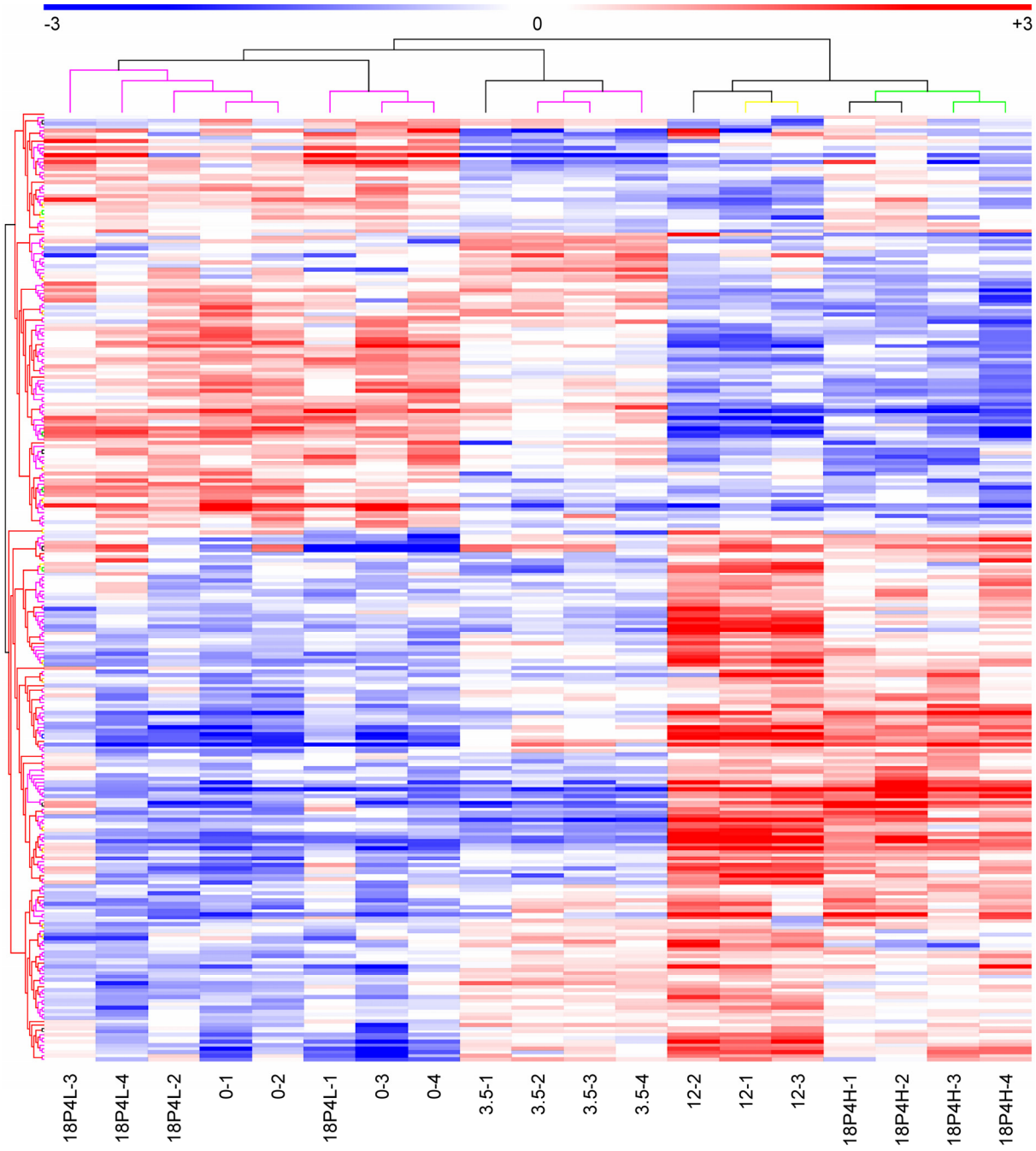
Watanabe H, Suzuki A, Kobayashi M, Takahashi E, Itamoto M, Lubahn DB, Handa H, Iguchi T (2003) Analysis of temporal changes in the expression of estrogen-regulated genes in the uterus. *J.Mol.Endocrinol.* **30**, 347-358.

White CA, Salamonsen LA (2005) A guide to issues in microarray analysis: application to endometrial biology. *Reproduction* **130**, 1-13.

Wolf E, Arnold GJ, Bauersachs S, Blum H, Frohlich T, Hiendleder S, Mitko K, Reichenbach HD (2006) Funktionale Genomforschung – neue Ansätze zur Verbesserung der Fruchtbarkeit beim Rind. *Züchtungskunde* **78**, 428-439.

Wolf E, Arnold GJ*, et al.* (2003) Embryo-maternal communication in bovine - strategies for deciphering a complex cross-talk. *Reprod.Domest.Anim* **38**, 276-289.

10 Appendix



Supplemental Figure 1:

Hierarchical clustering of genes and samples. Mean centred values (log2 value of a gene minus mean of all 19 log2 values of this gene) of cDNAs with significant changes during the oestrous cycle were used for HCL support trees (MultiExperiment Viewer 4.0). Colour coding of the support trees: black: 100% support; grey: 90-100%, blue: 80-90%, green: 70-80%, yellow: 60-70%, orange: 50-60%, pink: 0-50%, red: 0% support (percentage of time a given node was supported over the resampling trials).

Supplemental Table 1:

All significant revealing differentially expressed genes, ordered according to cluster affiliation

Gene description of best BLAST hit	Gene name	Gene symbol	Acc. no. BLAST hit	Acc. no. BOE EST	Entrez Gene ID bta	Entrez Gene ID hsa	Relative expression					Score (d)	q-value (%)	Local FDR	Cluster
							Day 18P4L	Day 0	Day 3.5	Day 12	Day 18P4H				
Bos taurus isolate 32027 mitochondrion, complete genome	16SrRNA	16SrRNA	AY676873				1.3	1.0	1.3	2.5	2.4	0.37	1.02	7.46	1
Bos taurus aconitase 2, mitochondrial (ACO2)	aconitase 2, mitochondrial	ACO2	NM_173977	EB086479	280976	50	1.1	1.0	1.0	2.1	2.8	0.50	0.00	0.00	1
Bos taurus acetyl-Coenzyme A synthetase 2 (AMP forming)-like (ACAS2L)	acyl-CoA synthetase short-chain family member 1	ACSS1	NM_174746	EB086816	282873	84532	2.5	1.0	2.1	17.1	8.4	1.20	0.00	0.00	1
Bos taurus aldose reductase (AKR1B1)	aldo-keto reductase family 1, member B1 (aldose reductase)	AKR1B1	NM_001012519		317748	231	1.5	1.0	1.1	5.2	1.8	0.61	0.00	0.00	1
Bos taurus Rho GDP dissociation inhibitor (GDI) beta (ARHGDIB)	Rho GDP dissociation inhibitor (GDI) beta	ARHGDIB	NM_175797		327676	397	1.7	1.0	1.9	5.0	5.1	0.63	0.03	0.02	1
Homo sapiens ATPase, Na+/K+ transporting, beta 2 polypeptide (cDNA clone IMAGE:5733940), with apparent retained intron	ATPase, Na+/K+ transporting, beta 2 polypeptide	ATP1B2	BC035801	DV549142	282562	482	1.4	1.0	1.4	8.4	2.2	0.90	0.00	0.00	1
Homo sapiens ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9) (ATP5G3), nuclear gene encoding mitochondrial protein, transcript variant 2	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit C3 (subunit 9)	ATP5G3	NM_001689		540176	518	1.3	1.0	1.1	1.8	2.0	0.38	0.77	6.04	1
PREDICTED: Bos taurus similar to branched chain aminotransferase 1, cytosolic, transcript variant 1 (LOC505926)	branched chain aminotransferase 1, cytosolic	BCAT1	XM_582290		505926	586	1.8	1.0	1.1	6.1	2.9	0.61	0.00	0.00	1
PREDICTED: Bos taurus similar to Chromosome 14 open reading frame 73 (LOC615559)	chromosome 14 open reading frame 73	C17orf73	XM_687402	DV549110	615559	91828	1.3	1.0	1.0	3.0	2.0	0.43	0.24	1.17	1

PREDICTED: Bos taurus similar to Complement C4 precursor (LOC617689)	complement component 4A	C4A	XM_869995		280678	720	1.8	1.0	1.6	2.9	3.5	0.54	0.15	1.25	1
Bos taurus transcription factor RAM2 (CDCA7L)	cell division cycle 7 homolog	CDCA7L	NM_001104977	DV549229	514631	55536	1.1	1.0	1.1	2.2	1.9	0.61	1.02	8.32	1
PREDICTED: Bos taurus similar to cytoskeleton-associated protein 4 (LOC515784)	cytoskeleton-associated protein 4	CKAP4	XM_868540	EB086377	515784	10970	1.9	1.7	1.0	6.4	2.8	0.82	0.00	0.05	1
PREDICTED: Bos taurus similar to Chloride channel protein ClC-Kb (Chloride channel Kb) (ClC-K2) (LOC538145)	chloride channel Kb	CLCKNB	XM_618340		538145	1188	1.2	1.0	1.0	5.8	1.7	0.92	0.00	0.05	1
Bos taurus claudin-4 (CLDN4)	claudin-4	CLDN4	NM_001014391		414921	1364	1.7	1.2	1.0	2.8	3.3	0.51	0.00	0.00	1
PREDICTED: Bos taurus similar to CCR4-NOT transcription complex, subunit 1 isoform a (LOC533968)	CCR4-NOT transcription complex, subunit 1	CNOT1	XM_613555	DV549140	533968	23019	1.0	1.0	1.2	2.6	1.3	0.45	0.18	0.16	1
Bos taurus cystatin E/M (CST6)	cystatin E/M	CST6	NM_001012764		503685	1474	1.0	1.0	1.0	6.3	2.3	0.69	0.00	0.00	1
Bos taurus chemokine (C-X-C motif) receptor 4 (CXCR4)	chemokine (C-X-C motif) receptor 4	CXCR4	NM_174301	EB086565	281736	7852	1.0	1.1	1.3	2.0	2.2	0.36	1.02	8.52	1
PREDICTED: Bos taurus similar to Cytochrome P450 26A1 (Retinoic acid-metabolizing cytochrome) (P450RAI) (Retinoic acid 4-hydroxylase), transcript variant 1 (LOC539047)	cytochrome P450, family 26, subfamily A, polypeptide 1	CYP26A1	XM_584484		539047	1592	1.4	1.0	1.1	33.3	25.2	1.14	0.00	0.00	1
Homo sapiens cytochrome P450, family 39, subfamily A, polypeptide 1 (CYP39A1)	cytochrome P450, family 39, subfamily A, polypeptide 1	CYP39A1	NM_016593		511195	51302	1.1	1.0	1.2	8.3	5.0	0.92	0.00	0.00	1
Bos taurus putative diacylglycerol O-acyltransferase (DGAT2)	diacylglycerol O-acyltransferase homolog 2 (mouse)	DGAT2	NM_205793		404192	84649	1.0	1.2	1.2	17.1	1.8	1.02	0.00	0.01	1
PREDICTED: Bos taurus similar to deltex 3-like (LOC515051)	deltex 3-like (Drosophila)	DTX3L	XM_592997	DV936239	515051	151636	1.4	1.0	1.4	2.6	2.2	0.57	0.77	6.15	1
Contig57350, Chr.15, ENSBTAG00000004046	deltex 4 homolog (Drosophila)	DTX4	ENSBTAG00000004046	EB086367		23220	1.8	1.4	1.0	4.3	1.3	0.54	0.00	0.00	1
PREDICTED: Bos taurus similar to endothelial differentiation sphingolipid G-protein-coupled receptor 3, transcript variant 1 (LOC539123)	endothelial differentiation, sphingolipid G-protein-coupled receptor, 3	EDG3	XM_001252512	DV936182	539123	1903	1.1	1.0	1.2	2.4	1.9	0.46	0.10	0.00	1

Bos taurus similar to ephrin A1 isoform a precursor (MGC128366)	ephrin-A1	EFNA1	NM_001034292	DV549172	507319	1942	1.6	1.0	1.1	2.9	2.7	0.48	0.10	0.00	1
CR382918 Bovine multi-stage muscles library (bcaj) Bos taurus cDNA clone bcaj0005a.c.05 5prim sequence	endothelial PAS domain protein 1	EPAS1	CR382918	EB086495	282711	2034	1.6	1.4	1.0	2.1	2.8	0.39	0.61	4.36	1
PREDICTED: Bos taurus similar to expressed sequence AV312086, transcript variant 1 (LOC537453)	hypothetical protein FLJ36874	FLJ36874	XM_872692	EB086580	537453	219988	1.3	1.0	1.4	2.8	2.3	0.45	0.18	0.26	1
PREDICTED: Bos taurus similar to FXYD domain containing ion transport regulator 4, transcript variant 3 (LOC506918)	FXYD domain containing ion transport regulator 4	FXYD4	XM_583436		506918	53823	2.4	2.1	1.0	38.0	10.9	1.76	0.00	0.00	1
Bos taurus growth arrest and DNA-damage-inducible, gamma (GADD45G)	growth arrest and DNA-damage-inducible, gamma	GADD45G	NM_001045901	EB086401	504939	10912	1.6	1.2	1.0	4.8	3.1	0.87	0.00	0.06	1
PREDICTED: Bos taurus similar to UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferase (LOC616218)	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 2 (GalNAc-T2)	GALNT2	XM_868188		616268	2590	1.0	1.0	1.0	2.6	2.3	0.47	0.10	0.00	1
PREDICTED: Bos taurus similar to GM2 ganglioside activator protein (LOC504524)	GM2 ganglioside activator	GM2A	XM_580665	DV549156	504524	2760	1.1	1.0	1.7	9.2	3.2	0.90	0.00	0.05	1
Homo sapiens helicase with zinc finger domain (HELZ)	helicase with zinc finger	HELZ	NM_014877	DV549164		9931	1.4	1.1	1.0	3.1	2.6	0.67	0.00	0.00	1
Bos taurus isocitrate dehydrogenase 1 (NADP+), soluble (IDH1)	isocitrate dehydrogenase 1 (NADP+), soluble	IDH1	NM_181012	DV936319	281235	3417	1.2	1.6	1.0	5.3	3.9	0.76	0.00	0.00	1
Bos taurus isocitrate dehydrogenase 2 (NADP+), mitochondrial (IDH2)	isocitrate dehydrogenase 2 (NADP+), mitochondrial	IDH2	NM_175790	DV549177	327669	3418	1.4	1.0	1.4	4.7	2.0	0.68	0.00	0.00	1
PREDICTED: Bos taurus hypothetical LOC506759, transcript variant 2 (LOC506759); Contig61327, ChrUn.478	interferon, gamma-inducible protein 16	IFI16	XM_863928	DV936501	506759	3428	1.2	1.0	1.0	1.9	2.3	0.41	0.43	3.16	1
Bos taurus similar to putative ISG12(a) protein (ISG12(A))	interferon, alpha-inducible protein 27	IFI27	NM_001038050	DV936232	507138	3429	1.4	1.2	1.0	2.2	2.2	0.40	0.43	3.79	1
PREDICTED: Bos taurus similar to melanoma differentiation associated protein-5 (LOC535490)	interferon induced with helicase C domain 1	IFIH1	XM_615590	EB086701	535490	64135	2.0	1.2	1.0	6.2	2.0	0.69	0.00	0.00	1
Bos taurus similar to Interferon	interferon regulatory factor 6	IRF6	NM_001076934		614253	3664	1.6	1.0	1.3	5.3	4.5	0.83	0.00	0.06	1

regulatory factor 6 (IRF-6), (MGC143192)															
Bos taurus interferon-stimulated protein, 15 kDa (ISG15)	ISG15 ubiquitin-like modifier	ISG15	NM_174366	DV936411	281871	9636	1.3	1.0	1.1	2.8	1.9	0.44	0.32	1.84	1
1183530 MARC 7BOV Bos taurus cDNA 5' sequence; SCAFFOLD110056	kinase suppressor of ras 2	KSR2		DV549180		283455	1.2	1.3	1.0	2.5	2.3	0.42	0.34	1.90	1
Bos taurus legumain (LGMN)	legumain	LGMN	NM_174101	EB086855	281281	5641	1.2	1.0	1.3	3.6	2.4	0.47	0.12	0.45	1
UMC-bend_0B01-024-d11 Day 16 Uterus from a pregnant animal bend Bos taurus cDNA 3' sequence	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	LIPA	CO728274	DV936145		3988	2.3	1.4	1.0	6.3	2.3	0.71	0.00	0.00	1
PREDICTED: Bos taurus similar to LMBR1L protein (LOC539015)	limb region 1 homolog (mouse)-like	LMBR1L	XR_027670		539015	55716	1.0	1.0	1.2	1.8	1.8	0.41	0.43	3.13	1
PREDICTED: Homo sapiens similar to WDNM1-like protein (LOC650626)	similar to WDNM1 homolog	LOC650626	XM_939715	EB086745		650626	1.2	1.0	1.0	6.7	9.0	0.73	0.00	0.00	1
Contig38875, ChrUn.41	hypothetical protein LOC784115	LOC784115		DV936147	784115		3.1	1.0	1.0	9.3	23.0	0.86	0.00	0.06	1
PREDICTED: Bos taurus hypothetical protein LOC787439 (LOC787439)	hypothetical protein LOC787439	LOC787439	XM_001254835	DV936226	787439		1.6	1.3	1.0	1.5	2.2	0.36	1.02	8.41	1
Mus musculus low density lipoprotein receptor-related protein 2 (cDNA clone IMAGE:4974943), partial cds	low density lipoprotein receptor-related protein 2	LRP2	BC040788	DV549139		4036	1.0	1.3	1.0	6.1	2.4	0.53	0.00	0.00	1
PREDICTED: Bos taurus similar to Lymphocyte antigen 6 complex locus G6C protein precursor (LOC505805)	lymphocyte antigen 6 complex, locus G6C	LY6G6C	NM_001077856		505805	80740	1.9	1.0	1.3	55.9	15.6	1.29	0.00	0.00	1
PREDICTED: Bos taurus similar to lymphocyte antigen 6 complex, locus G6E, transcript variant 1 (LOC512743)	lymphocyte antigen 6 complex, locus G6E	LY6G6E	NM_024123	DV549163	512743	79136	1.2	1.0	1.2	10.9	3.8	0.48	0.10	0.00	1
Bos taurus similar to Melanoma-associated antigen D2 (MAGE-D2 antigen) (MAGE-D) (Breast cancer associated gene 1 protein) (BCG-1) (11B6) (Hepatocellular carcinoma associated protein JCL-1) (MGC139951)	melanoma antigen family D, 2	MAGED2	NM_001075665		514372	10916	1.0	1.0	1.2	1.4	2.1	0.45	0.18	0.08	1

000929BEMN030540HT BEMN Bos taurus cDNA sequence; Homo sapiens mitogen-activated protein kinase 1 (MAPK1), transcript variant 1	mitogen-activated protein kinase 1	MAPK1	DY168238		327672	5594	1.0	1.0	1.2	3.2	6.8	1.03	0.00	0.01	1
PREDICTED: Bos taurus similar to N-benzoyl-L-tyrosyl-p-amino-benzoic acid hydrolase beta subunit, transcript variant 1 (LOC540701)	meprin A, beta	MEP1B	XM_612703		540701	4225	1.5	1.0	2.2	12.2	14.7	0.75	0.00	0.00	1
Bos taurus membrane spanning 4-domain subfamily A member 8B (ms4A8B)	ms4A8B protein	MS4A8B	NM_001034056		415111	83661	1.8	1.2	1.0	5.2	5.1	0.62	0.00	0.00	1
Bos taurus interferon-inducible myxovirus resistance-1 protein (Mx1) gene, Mx1-a allele, exon 15 and complete cds	myxovirus (influenza) resistance 1, (murine homolog)	MX1	AY251202		280872	4599	1.3	1.0	1.2	2.3	2.0	0.38	0.82	6.77	1
Bos taurus similar to N-myc downstream regulated gene 4 (MGC140660)	NDRG family member 4	NDRG4	NM_001075695	DV549184	515033	65009	1.2	1.4	1.0	4.7	1.8	0.64	0.00	0.00	1
968490 MARC 4BOV Bos taurus cDNA 3'	nebulette	NEBL	CK846036	DV549174		10529	1.2	1.1	1.0	4.5	2.2	0.71	0.00	0.00	1
PREDICTED: Bos taurus similar to Sialidase 1 precursor (Lysosomal sialidase) (N-acetyl-alpha-neuraminidase 1) (Acetylneuraminyl hydrolase) (G9 sialidase) (LOC505554)	sialidase 1 (lysosomal sialidase)	NEU1	XM_581856		505554	4758	1.4	1.4	1.0	1.7	2.3	0.38	0.77	6.81	1
Bos taurus Niemann-Pick disease, type C1 (NPC1)	Niemann-Pick disease, type C1	NPC1	NM_174758		286772	4864	1.3	1.0	1.2	2.3	2.0	0.50	0.00	0.00	1
Bos taurus 2',5'-oligoadenylate synthetase 1, 40/46kDa (OAS1)	2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	NM_001040606	DV936307	654488	4938	1.1	1.0	1.1	2.4	1.9	0.40	0.43	3.77	1
Bos taurus similar to phosphatidylethanolamine-binding protein 4 (MGC127772)	phosphatidylethanolamine-binding protein 4	PEBP4	NM_001034440		513254	157310	1.6	1.0	1.1	4.0	2.1	0.54	0.02	0.00	1
Bos taurus proenkephalin (PENK)	proenkephalin	PENK	NM_174141	DV936200	281387	5179	1.5	1.0	1.2	24.8	45.1	1.51	0.00	0.01	1

PREDICTED: Bos taurus similar to Group 10 secretory phospholipase A2 precursor (Group X secretory phospholipase A2) (Phosphatidylcholine 2-acylhydrolase GX) (GX sPLA2) (sPLA2-X), transcript variant 1 (LOC613966)	phospholipase A2, group X	PLA2G10	XM_864950	DV549157	613966	8399	2.4	1.0	2.1	8.1	3.9	0.73	0.00	0.00	1
Tissue plasminogen activator	plasminogen activator, tissue	PLAT	X85800		281407	5327	1.9	1.2	1.0	2.1	2.0	0.37	1.02	7.78	1
Bos taurus retinoic acid receptor responder (tazarotene induced) 2 (RARRES2)	retinoic acid receptor responder (tazarotene induced) 2	RARRES2	NM_001046020	DV549112	508990	5919	1.4	1.1	1.0	5.1	5.8	1.01	0.00	0.01	1
Bos taurus receptor accessory protein 6 (REEP6)	receptor accessory protein 6	REEP6	NM_001046605	DV936150	617543	92840	1.3	1.0	1.1	2.7	1.7	0.37	0.77	7.17	1
PREDICTED: Bos taurus similar to RalBP1 associated Eps domain containing protein 1 (RalBP1-interacting protein 1), transcript variant 1 (LOC536155)	RALBP1 associated Eps domain containing 1	REPS1	XM_616276		536155	85021	1.4	1.0	1.5	3.0	2.2	0.48	0.34	2.65	1
BP111960 ORCS bovine utero-placenta cDNA Bos taurus cDNA clone ORCS12550 5'	ribosomal protein L36a	RPL36A	BP111960		508165	6173	1.7	2.1	1.0	3.9	1.5	0.39	0.61	4.42	1
Bos taurus reticulon 3 (RTN3)	reticulon 3	RTN3	NM_182657		359721	10313	1.0	1.1	1.0	2.2	1.5	0.38	0.77	6.69	1
Bos taurus selenoprotein P-like protein precursor (SEPP1)	selenoprotein P-like protein precursor	SEPP1	NM_174459	DV549257	282066	6414	1.2	1.0	1.4	3.5	3.5	0.69	0.00	0.03	1
Bos taurus serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary) (SERPING1)	serine (or cysteine) proteinase inhibitor, clade G (C1 inhibitor), member 1, (angioedema, hereditary)	SERPING1	NM_174821	EB086646	281035	710	1.9	1.0	1.9	5.8	4.0	0.72	0.00	0.01	1
Homo sapiens SH3 multiple domains 1 (SH3MD1)	SH3 and PX domains 2A	SH3PXD2A	NM_014631			9644	1.6	1.0	1.7	3.3	3.9	0.75	0.00	0.00	1
LB0024.CR_A11 GC_BGC-02 Bos taurus cDNA clone IMAGE:7898005 5' sequence	solute carrier family 1 (neuronal/epithelial high affinity glutamate transporter, system Xag), member 1	SLC1A1	DN825839			6505	1.7	1.0	1.4	3.6	4.1	0.44	0.24	0.45	1
Bos taurus SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1 (SMARCB1)	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1	SMARCB1	NM_001040557	DV549104	537412	6598	1.2	1.3	1.0	5.9	9.1	1.09	0.00	0.00	1

Bos taurus teratocarcinoma-derived growth factor 1 (TDGF1)	teratocarcinoma-derived growth factor 1	TDGF1	NM_001080358	DV549151	784029	6997	1.1	1.0	1.0	28.0	1.4	1.10	0.00	0.00	1
PREDICTED: Bos taurus similar to LOC443715 protein, transcript variant 1 (LOC615277)	thioesterase superfamily member 4	THEM4	XM_589824	DV936288	615277	117145	1.8	1.6	1.0	2.1	5.4	0.44	0.24	0.55	1
PREDICTED: Bos taurus similar to LOC443715 protein, transcript variant 1 (LOC615277)	thioesterase superfamily member 5	THEM5	XM_589824		615277	284486	6.3	1.0	1.0	14.9	43.0	0.76	0.14	1.15	1
Bos taurus tissue inhibitor of mettaloproteinase 2 (TIMP2)	tissue inhibitor of mettaloproteinase 2	TIMP2	NM_174472		282093	7077	1.8	1.4	1.0	8.9	2.8	0.92	0.00	0.02	1
PREDICTED: Bos taurus similar to oxidized-LDL responsive gene 2, transcript variant 2 (LOC509642)	tubulointerstitial nephritis antigen-like 1	TINAGL1	XM_882308		509642	64129	2.3	1.0	1.0	2.5	3.2	0.36	1.02	9.38	1
Ovis aries UDP-glucuronosyltransferase (SHEUGT1A07)	UDP glucuronosyltransferase 1 family, polypeptide A6	UGT1A6	NM_001009189			54578	1.9	1.0	1.5	5.1	3.4	0.55	0.00	0.00	1
Reptig1015998, ChrUn.10273	unknown	unknown					1.3	1.0	1.6	9.1	13.0	0.90	0.00	0.05	1
PREDICTED: Bos taurus similar to KIAA1404 protein (LOC539807)	zinc finger, NFX1-type containing 1	ZNFX1	XM_589621	EB086754	539807	57169	2.2	1.0	1.3	8.5	3.4	0.70	0.09	0.16	1
4082241 BARC 9BOV Bos taurus cDNA clone 9BOV20_L10 3'	ATP-binding cassette, sub-family G (WHITE), member 1	ABCG1	CK966960	DV549193		9619	1.2	1.0	1.9	4.4	1.7	0.54	0.00	0.00	2
PREDICTED: Bos taurus similar to Y55F3AM.10 (LOC615289)	abhydrolase domain containing 14B	ABHD14B	XM_867054	DV549126	615289	84836	1.1	1.0	2.0	3.0	1.5	0.60	0.00	0.00	2
PREDICTED: Bos taurus similar to ankyrin repeat and BTB (POZ) domain containing 1 (LOC507417)	ankyrin repeat and BTB (POZ) domain containing 1	ABTB1	XM_584015	DV549205	507417	80325	1.2	1.0	2.7	3.3	3.8	0.64	0.00	0.00	2
757911 MARC 6BOV Bos taurus cDNA 3' sequence	angiotensinogen (serine (or cysteine) proteinase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 8)	AGT	CB533310	DV549136	527114	183	1.5	1.0	3.4	14.5	6.6	0.91	0.00	0.05	2
Bos taurus alkaline phosphatase, liver/bone/kidney (ALPL)	alkaline phosphatase, liver/bone/kidney	ALPL	NM_176858		280994	249	1.8	1.0	2.5	6.7	3.2	0.64	0.05	0.02	2
Bos taurus annexin A8 (ANXA8)	annexin A8	ANXA8	NM_174241		281627	244	1.2	1.0	2.1	2.6	1.7	0.41	0.48	2.97	2
Bos taurus beta-2-microglobulin (B2M)	beta-2-microglobulin	B2M	NM_173893		280729	567	1.7	1.0	1.7	2.2	1.5	0.38	0.77	6.15	2
Bos taurus BAI1-associated protein 2-like 2 (BAIAP2L2)	BAI1-associated protein 2-like 2	BAIAP2L2	NM_001035498		617924	80115	1.2	1.0	2.8	8.9	4.8	0.89	0.00	0.00	2
Chr.19 50940107-50940208; Bos taurus similar to CG8841-PA, isoform A (MGC139628)	chromosome 17 open reading frame 28	C17orf28	NM_001076924		540436	283987	1.3	1.0	1.5	2.8	1.7	0.50	0.00	0.00	2

Bos taurus complement component 1, r subcomponent (C1R)	complement component 1, r subcomponent	C1R	NM_001034407		511581	715	1.9	1.0	2.5	2.8	3.8	0.45	0.31	2.10	2
Homo sapiens mRNA; cDNA DKFZp761M0111 (from clone DKFZp761M0111)	chromosome 6 open reading frame 155	C6orf155	AL137346	DV549169		79940	1.0	1.1	2.0	2.8	1.7	0.50	0.00	0.00	2
PREDICTED: Bos taurus similar to Calpain 5, transcript variant 1 (LOC536988)	calpain 5	CAPN5	XM_617141		536988	726	1.0	1.0	1.6	1.8	1.4	0.38	0.77	6.36	2
Bos taurus cyclin B1 (CCNB1)	cyclin B1	CCNB1	NM_001045872	DV549111	327679	891	1.0	1.4	2.7	4.7	1.3	0.52	0.15	1.05	2
Bos taurus cyclin D3 (CCND3)	cyclin D3	CCND3	NM_001034709		540547	896	2.0	1.0	4.2	8.8	5.4	0.71	0.00	0.00	2
Bos taurus similar to CD74 antigen isoform b (MGC127643)	CD74 antigen (invariant polypeptide of major histocompatibility complex, class II antigen-associated)	CD74	NM_001034735	EB086792	281059	972	1.9	1.0	3.4	2.5	2.3	0.51	0.00	0.00	2
Bos taurus CD81 antigen (target of antiproliferative antibody 1) (CD81)	CD81 molecule	CD81	NM_001035099		511435	975	1.0	1.3	2.1	2.1	1.4	0.42	0.79	4.88	2
PREDICTED: Bos taurus hypothetical LOC516405, transcript variant 1 (LOC516405)	carboxymethylenebutenolidase homolog (Pseudomonas)	CMBL	XM_594558	DV549147	516405	134147	1.0	1.1	1.5	2.5	1.5	0.45	0.18	0.16	2
Mus musculus cyclin M3 (Cnnm3), transcript variant 2	cyclin M3	CNNM3	NM_053186	EB086820		26505	1.0	1.0	1.8	2.1	2.1	0.50	0.00	0.00	2
Homo sapiens collagen, type V, alpha 3 (COL5A3)	collagen, type V, alpha 3	COL5A3	NM_015719			50509	1.0	1.5	2.0	3.1	1.5	0.53	0.00	0.00	2
PREDICTED: Bos taurus similar to cryptochrome 2 (photolyase-like) (LOC509058)	cryptochrome 2 (photolyase-like)	CRY2	XM_585942	EB086714	509058	1408	1.4	1.0	2.5	4.6	2.6	0.78	0.00	0.02	2
PREDICTED: Bos taurus similar to Cytochrome P450 27, mitochondrial precursor (Cytochrome P-450C27/25) (Sterol 26-hydroxylase) (Sterol 27-hydroxylase) (Vitamin D(3) 25-hydroxylase) (5-beta-cholestane-3-alpha,7-alpha,12-alpha-triol 27-hydroxylase), transcript variant 1 (LOC511960)	cytochrome P450, family 27, subfamily A, polypeptide 1	CYP27A1	XM_589389		511960	1593	1.0	1.0	2.0	5.7	3.1	0.87	0.00	0.06	2
Chr.16 27117065-27117459; nr-, est-, repetetiv, locus for similar to flavin containing monooxygenase 4 (H.s.)	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	NM_007034			11080	1.0	1.3	2.0	2.6	2.6	0.49	0.10	0.00	2
Homo sapiens eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	eukaryotic translation elongation factor 1 alpha 1	EEF1A1	NM_001402		282220	1915	1.1	1.0	1.5	2.1	2.3	0.40	0.61	3.89	2

PREDICTED: Bos taurus similar to ectonucleotide pyrophosphatase/phosphodiesterase 1 (LOC615535), partial mRNA	ectonucleotide pyrophosphatase/phosphodiesterase 1	ENPP1	XM_867376		615535	5167	1.1	1.0	2.3	4.6	2.8	0.62	0.00	0.00	2
Bos taurus similar to epoxide hydrolase 1 (MGC126963)	epoxide hydrolase 1, microsomal (xenobiotic)	EPHX1	NM_001034629		535293	2052	1.0	1.3	2.1	2.8	1.5	0.45	0.18	0.02	2
Bos taurus similar to epoxide hydrolase 2, cytoplasmic (MGC139743)	epoxide hydrolase 2, cytoplasmic	EPHX2	NM_001075534		511716	2053	1.0	1.1	2.0	5.0	2.2	0.69	0.00	0.03	2
BP162142 full-length enriched swine cDNA library, adult thymus Sus scrofa cDNA clone THY010001C04 5' sequence; SCAFFOLD215209	family with sequence similarity 53, member B	FAM53B	BP162142	EB086632		9679	1.3	1.0	1.9	2.3	3.1	0.44	0.24	0.47	2
4103922 BARC 9BOV Bos taurus cDNA clone 9BOV26_C17 5' sequencePREDICTED: Bos taurus similar to expressed sequence AV312086, transcript variant 1 (LOC537453) Contig50573, Chr.16	flavin containing monooxygenase 4	FMO4	CK973468		537453	2329	1.1	1.0	3.2	7.7	5.6	0.70	0.00	0.00	2
1459642 MARC 7BOV Bos taurus cDNA 5' sequence; Contig70678, Chr.5, ENSBTAG00000000015	FAD-dependent oxidoreductase domain containing 2	FOXRED2	DT897387			80020	2.8	2.4	4.1	6.7	1.0	0.62	0.00	0.00	2
Bos taurus GABA(A) receptor-associated protein like 1 (GABARAPL1)	GABA(A) receptor-associated protein like 1	GABARAPL1	NM_001033616	EB086780	338472	23710	1.7	1.0	1.9	2.5	2.3	0.40	0.61	4.25	2
Homo sapiens chromosome 7 genomic contig, reference assembly; Contig3104, Chr.4, ENSBTAG00000020228	HECT, C2 and WW domain containing E3 ubiquitin protein ligase 1	HECW1	NT_007819		514243	23072	1.2	1.0	2.2	3.2	3.3	0.47	0.10	0.00	2
Homo sapiens cDNA clone MGC:134704 IMAGE:40022212, complete cds	HFM1, ATP-dependent DNA helicase homolog (S. cerevisiae)	HFM1	BC114377			164045	2.0	1.0	2.5	3.5	3.0	0.37	0.77	6.85	2
Bos taurus isolate 23_24+_+F05.1 MHC class I antigen (Duffy, Crowe *et al.*) gene, partial cds	major histocompatibility complex, class I, C	HLA-C	DQ121198		515712	3107	1.8	1.0	2.0	3.1	2.1	0.41	0.44	3.22	2
PREDICTED: Bos taurus similar to heterogeneous nuclear ribonucleoprotein L isoform a, transcript variant 4 (LOC504520)	heterogeneous nuclear ribonucleoprotein L	HNRPL	XM_580661		504520	3191	1.8	1.0	1.8	2.7	2.5	0.39	0.61	4.53	2

Bos taurus similar to putative 6-16 protein (6-16)	interferon, alpha-inducible protein 6	IFI6	NM_001075588	DV936154	512913	2537	1.3	1.0	2.1	3.6	1.6	0.48	0.27	2.00	2
interferon responsive factor 1	interferon responsive factor 1	IRF1	NM_177432		337917	3659	1.5	1.0	2.0	2.5	3.4	0.48	0.10	0.00	2
Bos taurus interferon-stimulated transcription factor 3, gamma 48kDa (ISGF3G)	interferon-stimulated transcription factor 3, gamma 48kDa	ISFG3G	NM_001024506		509855	10379	1.1	1.0	1.4	2.0	1.7	0.39	0.61	4.86	2
PREDICTED: Bos taurus similar to integrin beta 4 isoform 3 precursor, transcript variant 1 (LOC506995)	integrin, beta 4	ITGB4	XM_583533	DV549165	506995	3691	1.1	1.0	1.5	3.3	2.2	0.57	0.00	0.00	2
Bos taurus integral membrane protein 2B (ITM2B)	integral membrane protein 2B	ITM2B	NM_001035093		510575	9445	1.0	1.1	1.7	1.6	1.6	0.42	0.30	2.00	2
PREDICTED: Bos taurus similar to colon Kruppel-like factor, transcript variant 3 (LOC535702)	Kruppel-like factor 5 (intestinal)	KLF5	XM_866267		535702	688	1.0	1.0	2.0	2.6	2.4	0.48	0.10	0.00	2
PREDICTED: Bos taurus similar to KIAA0342 protein (LOC509859)	lupus brain antigen 1	LBA1	XR_028825	DV936435	509859	9881	1.0	1.3	2.3	1.9	1.6	0.48	0.10	0.00	2
Bos taurus similar to Galectin-3 binding protein precursor (Lectin galactoside-binding soluble 3 binding protein) (Mac-2 binding protein) (Mac-2 BP) (MAC2BP) (Tumor-associated antigen 90K) (MGC127786)	lectin, galactoside-binding, soluble, 3 binding protein	LGALS3BP	NM_001046316	EB086737	531137	3959	1.7	1.0	2.2	3.7	2.6	0.41	0.43	3.03	2
Bos taurus likely ortholog of mouse D11lgp2 (LGP2)	likely ortholog of mouse D11lgp2	LGP2	NM_001015545		508378	79132	1.5	1.0	2.1	3.4	2.7	0.58	0.00	0.00	2
Bos taurus similar to Lymphocyte antigen Ly-6E precursor (Retinoic acid-induced gene E protein) (RIG-E) (Thymic shared antigen 1) (TSA-1) (Stem cell antigen 2) (SCA-2) (MGC140297)	lymphocyte antigen 6 complex, locus E	LY6E	NM_001046070	DV549155	510977	4061	1.2	1.0	1.5	4.1	1.8	0.58	0.00	0.00	2
Bos taurus methionine adenosyltransferase II, beta (MAT2B)	methionine adenosyltransferase II, beta	MAT2B	NM_001046526		614177	27430	1.0	1.2	1.7	1.9	1.7	0.38	0.77	5.91	2
Bos taurus clone RP42-135P2, complete sequence	met proto-oncogene (hepatocyte growth factor receptor)	MET	AC087860	EB086573	280855	4233	1.0	1.1	1.4	1.6	2.4	0.38	0.77	6.36	2
Bos taurus matrix Gla protein mRNA, complete cds	matrix Gla protein	MGP	AF210379		282660	4256	1.6	1.0	3.8	4.7	5.2	0.74	0.00	0.00	2
Bos taurus isolate 15_16+_+A06.1 MHC class I antigen (Duffy, Crowe *et al.*) gene, partial cds	MHC Class I antigen	MHC1	DQ121168				1.3	1.0	2.0	2.7	2.0	0.41	0.43	3.05	2

Bos taurus matrix metalloproteinase 2 (72 KDa type IV collagenase) (MMP2)	matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	MMP2	NM_174745	DV549221	282872	4313	1.4	1.2	3.5	1.4	1.0	0.39	0.61	5.37	2
Contig36704, ChrUn.74, ENSBTAG00000000991	mitochondrial ribosomal protein L32	MRPL32				64983	1.8	1.0	2.5	3.2	5.6	0.64	0.00	0.00	2
Bos taurus mitochondrial ribosomal protein S36 (MRPS36)	mitochondrial ribosomal protein S36	MRPS36	NM_001035438		613835	92259	1.5	1.3	2.2	6.2	1.0	0.67	0.00	0.00	2
Homo sapiens myotubularin related protein 3 (MTMR3), transcript variant 1	myotubularin related protein 3	MTMR3	NM_153050	DV549128	281924	8897	2.0	1.0	3.7	18.5	9.6	1.45	0.00	0.00	2
Bos taurus neuronatin (cDNA clone MGC:128097 IMAGE:7989543), complete cds	neuronatin	NNAT	BC103128		353114	4826	1.0	1.5	4.0	2.7	1.3	0.45	0.18	0.02	2
PREDICTED: Bos taurus similar to Nuclear protein 1 (Protein p8) (Candidate of metastasis 1), transcript variant 1 (MGC128797)	nuclear protein 1; p8 protein (candidate of metastasis 1)	NUPR1	XM_867457	DV936172	614673	26471	1.0	2.2	2.9	4.0	2.5	0.51	0.05	0.00	2
Bos taurus similar to oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) isoform 1 precursor (MGC137985)	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	OGDH	NM_001076030		534599	4967	2.0	1.0	2.6	11.3	3.1	0.68	0.00	0.00	2
PREDICTED: Bos taurus similar to Poly [ADP-ribose] polymerase 10 (PARP-10) (LOC510991)	poly (ADP-ribose) polymerase family, member 10	PARP10	CK837739	EB086718	510991	84875	1.0	1.0	2.2	2.6	2.1	0.55	0.00	0.00	2
771635 MARC 6BOV Bos taurus cDNA 3' sequence	poly (ADP-ribose) polymerase family, member 12	PARP12	CB536605	EB086691	513185	64761	1.2	1.0	1.8	2.2	1.7	0.40	0.66	4.57	2
Bos taurus similar to poly(rC) binding protein 2 (MGC127996)	poly(rC) binding protein 2	PCBP2	NM_001034711		540653	5094	1.1	1.0	2.1	1.6	1.1	0.45	0.18	0.07	2
4100874 BARC 10BOV Bos taurus cDNA clone 10BOV7_D17 5'	PCI domain containing 2	PCID2	CK960198	DV549118	617943	55795	1.0	1.1	2.4	2.7	2.1	0.52	0.00	0.00	2
Bos taurus anti-oxidant protein 2 (non-selenium glutathione peroxidase, acidic calcium-independent phospholipase A2) (AOP2)	peroxiredoxin 6	PRDX6	NM_174643		282438	9588	1.2	1.0	1.7	2.5	1.4	0.44	0.24	0.54	2
Bos taurus proteasome (prosome, macropain) inhibitor subunit 1 (PI31) (PSMF1)	proteasome (prosome, macropain) inhibitor subunit 1 (PI31)	PSMF1	NM_001076478	EB086753	617807	9491	1.5	1.0	2.1	2.7	2.3	0.46	0.10	0.00	2

Bos taurus similar to Diamine acetyltransferase 1 (Spermidine/spermine N(1)-acetyltransferase 1) (SSAT) (SSAT-1) (Putrescine acetyltransferase) (Polyamine N-acetyltransferase 1) (MGC127572)	spermidine/spermine N1-acetyltransferase 1	SAT1	NM_001034333		508861	6303	1.2	1.0	1.5	3.0	1.4	0.44	0.39	3.03	2
Homo sapiens clone DNA176108 scavenger receptor hlg (UNQ2938) mRNA, complete cds	scavenger receptor class A, member 5 (putative)	SCARA5	DV549113			286133	1.2	1.0	7.1	17.9	18.2	1.29	0.00	0.00	2
Bos taurus similar to solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2 (MGC138014)	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	SLC11A2	NM_001046121		512464	4891	2.3	1.0	2.4	7.7	4.8	0.70	0.00	0.01	2
PREDICTED: Bos taurus hypothetical LOC618274 (LOC618274)	solute carrier family 16 (monocarboxylic acid transporters), member 11	SLC16A11	XR_028672	DV549159	618274	162515	1.9	1.0	4.0	16.9	8.5	1.32	0.00	0.00	2
Homo sapiens SRY (sex determining region Y)-box 17 (SOX17)	SRY (sex determining region Y)-box 17	SOX17	NM_022454			64321	1.0	1.1	2.4	4.8	2.0	0.75	0.00	0.00	2
Homo sapiens Sp3 transcription factor (SP3)	Sp3 transcription factor	SP3	NM_003111			6670	1.8	1.0	2.3	3.4	2.3	0.36	1.02	9.36	2
Bos taurus transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase) (TGM2)	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	TGM2	NM_177507	DV549153	281528	7052	2.0	1.0	2.9	10.6	5.8	0.69	0.00	0.02	2
PREDICTED: Bos taurus tight junction protein 1 (zo1), partial mRNA	tight junction protein 1 (zona occludens 1)	TJP1	XM_582218		407102	7082	1.5	1.0	2.9	3.5	2.2	0.46	0.18	0.00	2
55147 MARC 1BOV Bos taurus cDNA 5'	transmembrane protein 146	TMEM146	AW417843	DV549291	617472	257062	1.0	1.5	2.4	2.1	2.7	0.45	0.18	0.25	2
Homo sapiens TSC22 domain family, member 3 (TSC22D3), transcript variant 1	TSC22 domain family, member 3	TSC22D3	NM_198057			1831	1.0	1.0	1.5	4.7	2.6	0.76	0.00	0.02	2

Bos taurus similar to Tetraspanin-7 (Tspan-7) (Transmembrane 4 superfamily member 2) (Cell surface glycoprotein A15) (T-cell acute lymphoblastic leukemia associated antigen 1) (TALLA-1) (Membrane component, X chromosome, surface marker 1) (CD231 antigen)... (MGC139832)	tetraspanin 7	TSPAN7	NM_001076384		282383	7102	1.0	1.1	1.7	3.1	3.6	0.70	0.00	0.00	2
PREDICTED: Bos taurus similar to TXNIP, transcript variant 1 (LOC506790)	thioredoxin interacting protein	TXNIP	XM_583286	EB086788	506790	10628	1.3	1.0	2.8	3.5	3.7	0.58	0.00	0.00	2
Bos taurus similar to WAP four-disulfide core domain protein 2 precursor (Major epididymis-specific protein E4) (Epididymal secretory protein E4) (Putative protease inhibitor WAP5) (MGC127126)	WAP four-disulfide core domain 2	WFDC2	NM_001076490		618044	10406	1.0	1.2	3.2	2.2	1.4	0.46	0.10	0.00	2
Bos taurus XIAP associated factor-1 (BIRC4BP)	XIAP associated factor-1	XAF1	NM_001035075	DV936301	509740	54739	1.1	1.0	1.6	1.9	1.5	0.36	1.02	8.98	2
Homo sapiens cDNA FLJ34585 fis, clone KIDNE2008758; Contig146999, ChrUn.1825, ENSBTAG00000011928	zinc finger and BTB domain containing 20	ZBTB20	AK091904	EB086567		26137	1.0	1.2	1.9	1.9	1.9	0.40	0.43	3.49	2
Bos taurus similar to Actin, aortic smooth muscle (Alpha-actin-2) (MGC127800)	actin, alpha 2, smooth muscle, aorta	ACTA2	NM_001034502	EB086528	281593	59	3.2	3.2	1.4	1.8	1.0	0.49	0.00	0.00	3
Bos taurus actin, gamma 2, smooth muscle, enteric (ACTG2)	actin, gamma 2, smooth muscle, enteric	ACTG2	NM_001013592	DV549238	281595	72	2.2	1.8	1.0	1.3	1.6	0.37	0.77	7.16	3
Bos taurus similar to Protein C10orf58 precursor (MGC128811)	chromosome 10 open reading frame 58	C10orf58	NM_001034599		534049	84293	6.6	5.9	1.0	1.1	3.3	0.52	0.00	0.00	3
Contig112791, ChrUn.3510, ENSBTAG00000009291	chromosome 20 open reading frame 26 [Homo sapiens]	C20orf26				26074	3.3	3.4	1.0	1.4	1.0	0.63	0.00	0.00	3
PREDICTED: Bos taurus similar to 8D6 antigen, transcript variant 1 (LOC505043)	CD320 molecule	CD320	XM_581266		505043	51293	2.6	2.1	1.0	1.2	1.1	0.57	0.00	0.00	3
PREDICTED: Bos taurus collagen, type IV, alpha 1 (COL4A1)	collagen, type IV, alpha	COL4A1	XM_580317		282191	1282	2.6	2.2	1.3	1.2	1.0	0.44	0.36	2.49	3
PREDICTED: Bos taurus similar to FLJ20273 protein, transcript variant 1 (LOC540831)	RNA-binding protein	FLJ20273	XM_613340	DV549106	540831	54502	1.5	2.5	1.2	1.4	1.0	0.42	0.30	2.18	3

PREDICTED: Bos taurus similar to folate receptor 1 precursor, transcript variant 1 (LOC516067)	folate receptor 1 (adult)	FOLR1	XM_594203		516067	2348	3.9	8.4	1.0	6.7	2.4	0.36	1.02	9.27	3
PREDICTED: Bos taurus similar to gastrin-releasing peptide isoform 2 preproprotein (LOC615323)	gastrin-releasing peptide	GRP	XM_867101	DV549218	280809	2922	4.7	3.8	1.0	2.0	1.7	0.53	0.00	0.00	3
Homo sapiens insulin-like growth factor binding protein 2, 36kDa (cDNA clone MGC:10918 IMAGE:3627826), complete cds	insulin-like growth factor binding protein 2, 36kDa	IGFBP2	BC004312	DV936267	282260	3485	4.8	2.3	1.0	1.4	2.0	0.45	0.18	0.25	3
Bos taurus insulin-like growth factor-binding protein 6 (IGFBP6)	insulin-like growth factor binding protein 6	IGFBP6	NM_001040495	DV549250	404186	3489	1.6	2.0	1.1	1.2	1.0	0.36	1.02	8.44	3
Bos taurus immunoglobulin IgA heavy chain constant region gene, partial cds	immunoglobulin heavy constant alpha	IGHA	AF109167		281242		1.8	1.8	1.0	1.4	1.8	0.46	0.10	0.00	3
Bos taurus myosin, light peptide 6, alkali, smooth muscle and non-muscle (MYL6)	myosin, light peptide 6, alkali, smooth muscle and non-muscle	MYL6	NM_175780		281341	4637	1.9	1.6	1.0	1.5	1.4	0.36	1.48	9.75	3
Bos taurus nascent-polypeptide-associated complex alpha polypeptide (NACA)	nascent-polypeptide-associated complex alpha polypeptide	NACA	NM_001014916	DV549303	513312	4666	1.7	1.8	1.0	1.4	1.2	0.42	0.30	1.94	3
Bos taurus nucleoside phosphorylase (NP)	nucleoside phosphorylase	NP	NM_001007818		493724	4860	2.7	2.1	1.0	2.6	2.4	0.41	0.43	2.88	3
Bos taurus neuropeptide Y (NPY)	neuropeptide Y	NPY	NM_001014845		504216	4852	8.9	26.3	1.2	1.0	1.0	0.98	0.00	0.02	3
Bos taurus similar to procollagen C-endopeptidase enhancer (MGC137826)	procollagen C-endopeptidase enhancer	PCOLCE	NM_001045888	DV549261	504471	5118	2.0	3.0	1.0	1.0	1.2	0.47	0.10	0.05	3
Bos taurus calbindin 3, (vitamin D-dependent calcium binding protein) (CALB3)	S100 calcium binding protein G	S100G	NM_174257	EB086449	281658	795	3.8	4.0	1.0	5.6	2.1	0.45	0.31	1.94	3
PREDICTED: Bos taurus similar to SPIN protein, transcript variant 1 (LOC541068)	spindlin 1	SPIN1	XM_614403		541068	10927	1.7	1.6	1.0	1.2	1.8	0.36	1.02	8.56	3
Homo sapiens signal sequence receptor, gamma (translocon-associated protein gamma) (SSR3)	signal sequence receptor, gamma (translocon-associated protein gamma)	SSR3	NM_007107			6747	1.7	2.0	1.0	1.5	1.2	0.39	0.61	5.38	3
Homo sapiens stanniocalcin precursor (STC) mRNA, complete cds	stanniocalcin 1	STC1	NM_003155		338078	6781	3.3	3.4	1.0	1.3	1.8	0.48	0.05	0.00	3

Bos taurus tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor) (TIMP1)	tissue inhibitor of metalloproteinase 1 (erythroid potentiating activity, collagenase inhibitor)	TIMP1	NM_174471		282092	7076	3.4	3.1	1.0	2.2	1.7	0.59	0.00	0.00	3
Bos taurus uterine milk protein precursor (LOC286871)	uterine milk protein precursor	UTMP	NM_174797	DV936167	286871		87.0	38.0	1.0	3.2	3.9	0.64	0.07	0.61	3
Bos taurus vacuolar protein sorting protein 25 (VPS25)	vacuolar protein sorting 25 (yeast)	VPS25	NM_001015657	DV549245	534750	84313	1.5	2.0	1.1	1.1	1.0	0.36	1.48	9.84	3
Bos taurus zinc finger and BTB domain containing 16 (ZBTB16)	zinc finger and BTB domain containing 16	ZBTB16	NM_001037476		534401	7704	2.3	2.5	1.0	1.1	1.6	0.43	0.24	0.98	3
Homo sapiens chromosome 10 clone RP11-472G21, complete sequence	zinc finger protein 564	ZNF564	AC073370	DV549278		163050	1.8	2.1	1.0	1.1	1.0	0.37	0.77	7.09	3
Bos taurus differentiated embryo chondrocyte expressed gene 1 (BHLHB2)	basic helix-loop-helix domain containing, class B, 2	BHLHB2	NM_001024929		506945	8553	6.5	4.5	1.8	1.0	3.4	0.54	0.00	0.00	4
BovGen_23410 normal cattle brain Bos taurus cDNA clone RZPDp1056B2138Q 5' sequence; Contig138689, Chr.11	carnitine acetyltransferase	CRAT	CO895085		512902	1384	2.6	4.1	1.5	1.0	1.6	0.61	0.00	0.00	4
Bos taurus cathepsin K preproprotein (CTSK)	cathepsin K (pycnodysostosis)	CTSK	NM_001034435	DV549304	513038	1513	2.2	3.2	1.5	1.0	1.9	0.51	0.00	0.00	4
PREDICTED: Bos taurus similar to dynein, axonemal, heavy polypeptide 9 isoform 2 (LOC617408)	dynein, axonemal, heavy polypeptide 9	DNAH9	XM_597805		497209	1770	2.0	3.1	1.5	1.0	1.5	0.56	0.00	0.00	4
Bos taurus similar to guanylate-binding protein 5 (MGC142842)	guanylate binding protein 5	GBP5	NM_001075746	DV936326	516949	115362	1.9	2.1	1.6	1.0	2.0	0.37	1.02	8.06	4
Bos taurus heat shock 90kD protein 1, alpha (HSPCA)	heat shock protein 90kDa alpha (cytosolic), class A member 1	HSP90AA1	NM_001012670	DV549307	281832	3320	2.2	1.8	1.5	1.0	2.2	0.39	0.61	4.98	4
Bos taurus tumor rejection antigen (gp96) 1 (TRA1)	heat shock protein 90kDa beta (Grp94), member 1	HSP90B1	NM_174700		282646	7184	2.7	3.2	1.7	1.0	2.3	0.40	0.43	3.43	4
PREDICTED: Bos taurus similar to tubulin, alpha 1, transcript variant 1 (LOC539882)	alpha tubulinalpha tubulin	K-ALPHA-1	XM_590059		539882	10376	3.6	3.7	1.9	1.0	2.3	0.58	0.00	0.00	4
PREDICTED: Bos taurus similar to mucin 16 (LOC528826)	mucin 16	MUC16	XM_607258		528826	94025	1.9	2.3	1.3	1.0	1.0	0.38	0.77	6.47	4
Chr.17 37693794-37693883, ENSBTAG00000014628	2'-5' oligoadenylate synthetase 2	OAS2		DV936330	529660	4939	1.8	1.9	1.5	1.0	1.9	0.38	0.77	5.77	4

Bos taurus procollagen-proline, 2-oxoglutarate 4-dioxygenase (P4HB)	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	P4HB	NM_174135		281373	5034	2.0	2.7	1.5	1.0	1.2	0.50	0.05	0.00	4
PREDICTED: Bos taurus similar to Proprotein convertase subtilisin/kexin type 5 precursor (Proprotein convertase PC5) (Subtilisin/kexin-like protease PC5) (PC6) (Subtilisin-like proprotein convertase 6) (SPC6) (LOC528098)	proprotein convertase subtilisin/kexin type 5	PCSK5	XM_868406	DV549237	616396	5125	3.9	8.8	1.4	1.0	1.2	1.01	0.00	0.01	4
Bos taurus glucose regulated protein 58kD (GRP58)	protein disulfide isomerase family A, member 3	PDIA3	NM_174333		281803	2923	3.0	2.8	1.4	1.0	1.9	0.58	0.00	0.00	4
Bos taurus protein disulfide isomerase-associated 4 (PDIA4)	protein disulfide isomerase related protein	PDIA4	NM_001045879		415110	9601	2.0	2.0	1.3	1.0	1.1	0.44	0.24	0.81	4
Bos taurus profilin 1 (PFN1)	profilin 1	PFN1	NM_001015592		513895	5216	2.2	2.5	1.3	1.0	1.6	0.43	0.24	0.98	4
Bos taurus ribosomal protein L5 (RPL5)	ribosomal protein L5	RPL5	NM_001035306		515238	6125	1.8	2.7	2.1	1.0	2.0	0.36	1.02	9.66	4
BP230006B10E12 Soares normalized bovine placenta Bos taurus cDNA clone BP230006B10E12 5' sequence	ribosomal RNA processing 1 homolog B (S. cerevisiae) [Homo sapiens]	RRP1B	AW461785		510240	23076	1.7	1.5	1.0	1.1	2.0	0.39	0.61	5.31	4
Bos taurus similar to Collagen-binding protein 2 precursor (Colligin 2) (Rheumatoid arthritis related antigen RA-A47) (Serpin H2) (MGC127397)	serine (or cysteine) proteinase inhibitor, clade H (heat shock protein 47), member 1, (collagen binding protein 1)	SERPINH1	NM_001046063		510850	871	2.8	3.0	1.5	1.0	1.1	0.49	0.33	2.66	4
Bovine preprotachykinin B gene, exon 7	tachykinin 3, neurokinin beta	TAC3	M14352	EB086480	281513	6866	3.6	3.2	1.4	1.0	1.4	0.68	0.00	0.00	4
Bos taurus similar to 30 kDa protein (MGC128026)	transmembrane protein 111	TMEM111	NM_001035056		508371	55831	2.8	3.1	1.4	1.0	1.3	0.58	0.00	0.00	4
Tumor Necrosis Factor Receptor 1	tumor necrosis factor receptor superfamily, member 1A	TNFRSF1A	NM_174674		282527	7132	7.4	6.9	2.8	1.0	2.0	0.78	0.00	0.01	4
PREDICTED: Bos taurus similar to Tropomyosin 3, transcript variant 1 (LOC512435)	tropomyosin 3	TPM3	XM_589950		497019	7170	2.0	2.3	1.2	1.0	1.0	0.50	0.00	0.00	4
Bos taurus hypothetical protein MGC139023 (MGC139023)	zinc finger protein 22 (KOX 15)	ZNF22	NM_001077108	DV549292	768051	7570	2.3	2.1	1.4	1.0	3.0	0.38	0.77	6.49	4
Bos taurus actin, beta (ACTB)	actin, beta	ACTB	NM_173979		280979	60	1.9	2.0	1.4	1.3	1.0	0.38	0.74	6.04	5

PREDICTED: Bos taurus similar to Angiopoietin-related protein 2 precursor (Angiopoietin-like 2), transcript variant 1 (LOC512019)	angiopoietin-like 2	ANGPTL2	XM_589455		512019	23452	2.6	3.2	2.3	1.0	1.2	0.45	0.18	0.00	5
Bos taurus annexin A2 (ANXA2)	annexin A2	ANXA2	NM_174716		282689	302	2.6	3.2	1.9	1.0	1.4	0.49	0.12	0.32	5
Bos taurus ADP-ribosylation factor 3 (ARF3)	ADP-ribosylation factor 3	ARF3	NM_001015571		510994	377	2.1	2.1	1.6	1.4	1.0	0.44	0.18	0.40	5
AAFC03019924, Chr.25, ENSBTAG00000018050	cerebellar degeneration-related protein 2, 62kDa	CDR2	AC092338			1039	2.8	3.5	1.8	1.0	1.2	0.61	0.00	0.00	5
Bos taurus claudin 10 isoform b (CLDN10)	claudin 10 isoform b	CLDN10	NM_001014857		506545	9071	14.1	14.8	7.2	1.3	1.0	1.03	0.00	0.02	5
PREDICTED: Bos taurus collagen, type XII, alpha 1, transcript variant 1 (COL12A1)	collagen, type XII, alpha 1	COL12A1	XM_611630	DV549301	359712	1303	4.6	8.8	3.5	1.0	1.8	0.78	0.00	0.01	5
PREDICTED: Bos taurus similar to alpha 1 type XV collagen precursor (LOC537532)	collagen, type XV, alpha 1	COL15A1	XM_617701	EB086518	537532	1306	3.3	3.1	2.2	1.1	1.0	0.60	0.00	0.00	5
Bos taurus collagen, type I, alpha 2 (COL1A2)	collagen, type I, alpha 2	COL1A2	NM_174520	DV936377	282188	1278	2.3	4.0	2.5	1.4	1.0	0.68	0.00	0.01	5
Bos taurus similar to Collagen alpha 1(Tanaka, Jaradat *et al.*) chain precursor (LOC510833)	collagen, type III, alpha 1 chain, fibril forming	COL3A1	NM_001076831	EB086432	282190	1281	2.6	4.0	2.3	1.5	1.0	0.38	0.77	5.89	5
PREDICTED: Bos taurus similar to alpha 3 type VI collagen isoform 3 precursor, transcript variant 1 (LOC530657)	collagen, type VI, alpha 3	COL6A3	XM_609132		530657	1293	3.9	3.9	1.8	1.3	1.0	0.55	0.08	0.42	5
Chr. 13, Contig26355, ENSBTAG00000012407	forkhead box A2	FOXA2				3170	5.5	5.8	2.7	1.0	1.7	0.82	0.00	0.05	5
Bos taurus insulin-like growth factor-binding protein 4 (IGFBP4)	insulin-like growth factor binding protein 4	IGFBP4	NM_174557	DV549236	282262	3487	2.7	2.7	2.2	1.4	1.0	0.39	0.61	4.93	5
Bos taurus lectin, galactoside-binding, soluble, 1 (galectin 1) (LGALS1)	lectin, galactoside-binding, soluble, 1 (galectin 1)	LGALS1	NM_175782	DV549275	326598	3956	2.6	4.0	2.1	1.1	1.0	0.72	0.00	0.00	5
PREDICTED: Bos taurus similar to Equ c1, transcript variant 1 (LOC513329)	similar to Equ c1	LOC513329	XM_590993	DV549223	513329		2.8	5.7	2.5	1.2	1.0	0.82	0.00	0.06	5
PREDICTED: Bos taurus similar to Leucine-rich repeats and calponin homology domain-containing protein 3 (LOC506150)	leucine-rich repeats and calponin homology (CH) domain containing 3	LRCH3	XM_582557		506150	84859	4.1	4.0	2.2	1.4	1.0	0.55	0.00	0.00	5

Bos taurus similar to melanoma antigen family D, 1 (MGC128691)	melanoma antigen, family D, 1	MAGED1	NM_001046125	DV549232	512562	9500	2.0	2.6	1.4	1.1	1.0	0.56	0.00	0.00	5
Homo sapiens cDNA FLJ37829 fis, clone BRSSN2006611, weakly similar to Homo sapiens mRNA for mucolipidin	mucolipin 3	MCOLN3	AK095148	DV549203	514345	55283	2.7	2.9	2.2	1.0	1.0	0.52	0.00	0.00	5
Bos taurus microfibril-associated glycoprotein-2 (MAGP2)	microfibrillar associated protein 5	MFAP5	NM_174386	DV549213	281908	8076	2.7	3.2	2.4	1.3	1.0	0.50	0.00	0.00	5
PREDICTED: Bos taurus myosin, light polypeptide kinase (MYLK), partial mRNA	myosin, light polypeptide kinase	MYLK	XM_590786	EB086410	338037	4638	1.8	3.2	1.8	1.3	1.0	0.47	0.22	1.73	5
Homo sapiens nuclear receptor co-repressor 2 (NCOR2)	nuclear receptor co-repressor 2	NCOR2	NM_006312	DV549228		9612	2.8	3.0	2.0	1.0	1.1	0.50	0.00	0.00	5
Bos taurus Norrie disease (pseudoglioma) (NDP)	Norrie disease (pseudoglioma)	NDP	NM_001046090	DV549286	511596	4693	3.0	7.3	2.6	1.2	1.0	0.84	0.00	0.05	5
PREDICTED: Bos taurus nephroblastoma overexpressed (NOV)	nephroblastoma overexpressed gene	NOV	XM_582063		280879	4856	7.7	14.5	4.1	1.0	2.1	0.86	0.00	0.05	5
LB01623.CR_H23 GC_BGC-16 Bos taurus cDNA clone IMAGE:8086441 5' sequence; Chr.22 14335158-14335617	oxytocin receptor	OXTR	DT814146		281371	5021	21.0	17.6	13.9	1.0	4.8	0.93	0.00	0.02	5
PREDICTED: Bos taurus similar to Pyruvate kinase, isozymes M1/M2 (Pyruvate kinase muscle isozyme) (Cytosolic thyroid hormone-binding protein) (CTHBP) (THBP1) (LOC512571)	pyruvate kinase, muscle	PKM2	XM_590109	DV549269	512571	5315	2.0	2.5	1.4	1.1	1.0	0.44	0.24	0.78	5
PREDICTED: Bos taurus similar to serum-inducible kinase, transcript variant 1 (LOC539449)	polo-like kinase 2 (Drosophila)	PLK2	XM_587229		539449	10769	4.9	5.1	3.2	1.0	1.4	0.63	0.00	0.00	5
Bos taurus similar to retinoic acid receptor responder (tazarotene induced) 1 (MGC142566)	retinoic acid receptor responder (tazarotene induced) 1	RARRES1	NM_001075430		510102	5918	3.5	5.8	3.0	1.0	1.4	0.81	0.03	0.02	5
Homo sapiens SAR1 protein (SAR1)	SAR1 gene homolog A (S. cerevisiae)	SAR1A	NM_020150		517171	56681	4.3	6.4	2.8	1.0	1.9	0.72	0.00	0.00	5
Bos taurus syndecan 2 (SDC2)	syndecan 2 (heparan sulfate proteoglycan 1, cell surface-associated, fibroglycan)	SDC2	NM_001034788	EB086339	615785	6383	2.8	4.3	1.9	1.0	1.3	0.78	0.00	0,01	5

Bos taurus similar to Translocon-associated protein, delta subunit precursor (TRAP-delta) (Signal sequence receptor delta subunit) (SSR-delta) (MGC133642)	signal sequence receptor, delta (translocon-associated protein delta)	SSR4	NM_001038503		504438	6748	2.1	2.1	1.6	1.4	1.0	0.40	0.61	4.28	5
Angiopoietin receptor tie2	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	TEK	X71424		280939	7010	9.5	9.0	3.6	1.0	2.3	0.73	0.00	0.00	5
PREDICTED: Bos taurus transmembrane prostate androgen-induced protein (TMEPAI)	transmembrane, prostate androgen induced RNA	TMEPAI	XM_001249927		617469	56937	3.5	4.3	2.9	1.1	1.0	0.84	0.00	0.02	5
Bos taurus thymosin, beta 10 (TMSB10)	thymosin, beta 10	TMSB10	NM_174623		282385	9168	1.8	2.5	1.7	1.2	1.0	0.46	0.10	0.00	5
Bos taurus similar to Tenascin precursor (TN) (Hexabrachion) (Cytotactin) (Neuronectin) (GMEM) (JI) (Miotendinous antigen) (Glioma-associated-extracellular matrix antigen) (GP 150-225) (Tenascin-C) (TN-C) (MGC140517)	tenascin C (hexabrachion)	TNC	NM_001078026		540664	3371	11.0	12.5	5.2	1.0	1.7	0.89	0.00	0.01	5
Bos taurus similar to thyroid hormone receptor interactor 6 (MGC128885)	thyroid hormone receptor interactor 6	TRIP6	NM_001035469		615869	7205	1.9	3.1	1.7	1.2	1.0	0.57	0.00	0.00	5
PREDICTED: Bos taurus similar to tubulin, alpha 1, transcript variant 1 (LOC539882)	tubulin, alpha 1	TUBA1	XM_590059	EB086372	504244	7277	3.0	2.9	2.1	1.0	1.6	0.55	0.00	0.00	5
Bos taurus similar to tubulin, beta 5 (MGC128415)	tubulin, beta polypeptide	TUBB	NM_001046549	DV549268	281555	203068	2.6	2.6	1.8	1.0	1.1	0.55	0.08	0.24	5
Bos taurus apolipoprotein E (APOE)	apolipoprotein E	APOE	NM_173991	DV549312	281004	348	2.9	2.7	2.7	1.0	1.1	0.45	0.27	1.75	6
PREDICTED: Bos taurus similar to SMAP-2b (LOC615490), partial mRNA	bromodomain containing 8	BRD8	XM_867313		615490	10902	2.7	1.5	1.8	1.3	1.0	0.41	0.30	2.53	6
Homo sapiens hypothetical protein FLJ14054 (FLJ14054)	chromosome 5 open reading frame 23	C5orf23	NM_024563	EB086552		79614	3.5	3.8	3.9	1.0	1.3	0.75	0.00	0.00	6
Homo sapiens collagen, type I, alpha 1 (COL1A1)	collagen, type I, alpha 1	COL1A1	NM_001034039	EB086390	282187	1277	2.6	4.7	3.0	1.8	1.0	0.72	0.00	0.01	6
PREDICTED: Bos taurus similar to Collagen, type VI, alpha 1, transcript variant 1 (LOC511422)	collagen, type VI, alpha 1	COL6A1	XM_588755	EB086516	282193	1291	2.1	2.0	1.9	1.3	1.0	0.39	0.61	5.33	6

Homo sapiens deiodinase, iodothyronine, type II (DIO2), transcript variant 1	deiodinase, iodothyronine, type II	DIO2	NM_013989	EB086556	494548	1734	3.1	4.3	4.5	1.0	1.0	0.72	0.00	0.00	6
Chr.21 22010999-22011118, locus for similar to fumarylacetoacetate hydrolase (fumarylacetoacetase) [Homo sapiens]	fumarylacetoacetate hydrolase (fumarylacetoacetase)	FAH	NM_000137		508724	2184	2.1	3.7	3.0	1.0	1.0	0.56	0.00	0.00	6
PREDICTED: Bos taurus similar to fibulin 1 isoform C precursor, transcript variant 2 (LOC514588)	fibulin 1	FBLN1	XM_882469	DV549206	514588	2192	2.4	2.4	2.9	1.0	1.1	0.52	0.00	0.00	6
PREDICTED: Bos taurus similar to fibroblast growth factor receptor-like 1 precursor (LOC532327)	fibroblast growth factor receptor-like 1	FGFRL1	XM_610839		532327	53834	1.9	2.6	2.5	1.5	1.0	0.46	0.18	0.00	6
PREDICTED: Bos taurus protease, serine, 11 (PRSS11)	HtrA serine peptidase 1	HTRA1	XM_612097	DV549249	282326	5654	2.4	3.2	3.3	1.5	1.0	0.48	0.34	2.74	6
PREDICTED: Bos taurus hypothetical LOC539467 (LOC539467), partial mRNA	hypothetical LOC539467	LOC539467	XM_587378		539467	91461	4.5	3.5	3.8	1.0	1.1	0.69	0.00	0.00	6
Bos taurus peroxiredoxin 2 (PRDX2)	peroxiredoxin 2	PRDX2	NM_174763	DV549240	286793	7001	3.4	3.5	2.9	1.8	1.0	0.54	0.00	0.00	6
PREDICTED: Bos taurus similar to Protein patched homolog 2 (PTC2) (LOC507948)	patched homolog 2 (Drosophila)	PTCH2	XM_584648	DV549226	507948	8643	2.1	3.5	3.5	1.6	1.0	0.47	0.10	0.00	6
Bos taurus secreted protein, acidic, cysteine-rich [osteonectin] (SPARC)	secreted protein, acidic, cysteine-rich [osteonectin]	SPARC	NM_174464		282077	6678	2.9	4.1	2.9	1.6	1.0	0.60	0.18	1.18	6
1404210 MARC 7BOV Bos taurus cDNA 3' sequence; Contig11724, Chr.21	unknown	unknown	DN740440				2.2	3.1	2.5	1.0	1.0	0.58	0.00	0.00	6
Bos taurus brain ribonuclease (BRB); most similar hsa protein: ribonuclease, RNase A family, 1 (pancreatic)	brain ribonuclease	BRB	NM_173891	EB086532	280720	6035	1.3	1.2	2.2	1.0	1.0	0.37	1.02	7.88	7
PREDICTED: Bos taurus desmin, transcript variant 1 (DES)	desmin	DES	XM_588095		280765	1674	2.4	1.9	3.4	1.6	1.0	0.44	0.24	0.53	7
PREDICTED: Bos Taurus hypothetical LOC539299, transcript variant 1 (LOC539299)	DNA polymerase-transactivated protein 6	DNAPTP6	XM_586302	EB086800	539299	26010	1.0	1.1	2.4	1.1	1.0	0.53	0.00	0.00	7
PREDICTED: Bos taurus similar to dystonin isoform 1eA, transcript variant 1 (LOC535297)	dystonin	DST	XM_001252266	EB086418	535297	667	1.6	1.4	2.1	1.0	1.0	0.38	0.77	6.62	7

Bos taurus estrogen receptor 1 alpha (ESR1) mRNA, partial sequence	estrogen receptor 1	ESR1	AY656813	EB086353	281145	2099	1.9	1.7	3.1	1.0	1.2	0.45	0.18	0.00	7
PREDICTED: Bos taurus similar to tumor endothelial marker 5 (LOC782287); Contig84952, Chr.27, ENSBTAG00000008814	G protein-coupled receptor 124 (tumor endothelial marker 5)	GPR124	XM_001250919		782287	25960	1.3	1.9	2.6	1.3	1.0	0.56	0.00	0.00	7
BP107321 ORCS bovine utero-placenta cDNA Bos taurus cDNA clone ORCS10659 3' sequence	immunoglobulin superfamily, member 1	IGSF1	BP107321			3547	1.2	1.7	2.0	1.7	1.0	0.42	0.30	1.93	7
PREDICTED: Bos taurus similar to laminin alpha 4 (LOC529670)	laminin, alpha 4	LAMA4	XM_608123		529670	3910	1.0	1.5	3.2	1.4	1.3	0.49	0.00	0.00	7
PREDICTED: Bos Taurus hypothetical LOC616148 (LOC616148); Contig59859, ChrUn.3223, ENSBTAG00000026111	hypothetical LOC616148 [Bos taurus]; limb bud and heart development homolog (mouse)	LBH	XM_868111		616148	81606	2.1	1.8	3.2	1.0	1.1	0.41	0.43	3.21	7
Bos taurus isolate 32027 mitochondrion, complete genome	mitochondrially encoded cytochrome b	MT-CYB	AY676873		3283889	4519	1.8	3.2	2.9	1.0	1.8	0.41	0.48	3.64	7
Homo sapiens ornithine decarboxylase antizyme 1 (OAZ1)	ornithine decarboxylase antizyme 1	OAZ1	NM_004152		531865	4946	1.0	1.2	2.0	1.0	1.1	0.44	0.18	0.35	7
Bos taurus vimentin (VIM)	vimentin	VIM	NM_173969	DV936193	280955	7431	2.0	2.3	3.0	1.4	1.0	0.55	0.06	0.11	7

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