Female infertility is an increasingly important problem in cattle, causing enormous costs and retarding genetic progress. Current attempts to improve fertility by genetic selection are inefficient due to the low heritability of the respective trait, i.e. the nonreturn-rate 90 days after first service (NRR 90). Thus novel phenotypic traits more closely related to fertility are urgently needed. Since a large proportion of pregnancy losses occur in the preimplantation period, the interaction between early embryos and their maternal environment is an attractive target for systematic investigations, which may uncover mechanisms underlying early embryonic death. Based on holistic transcriptome and proteome studies [Hiendleder et al. 2005, Wolf et al. 2006] we attempt to understand the quantitative biology of embryo maternal communication and the regulation of endometrial receptivity [Wolf et al. 2003]. A short-term goal is the development of array-based systems for the differential diagnosis of fertility problems and for evaluating the connection between metabolic disturbances and reproductive functions. A long-term goal is the identification of genetic variation affecting the fertility-related gene expression profiles in female reproductive tissues such as endometrium. The endometrium undergoes marked functional changes during estrous cycle and pregnancy. As the adjacent environment of the conceptus, it represents the maternal interface for embryo maternal communication, which is essential to maintain pregnancy. Transcriptome studies provide the unique opportunity to assess molecular profiles changing in response to endocrine or metabolic stimuli or to embryonic pregnancy recognition signals [reviewed in Bauersachs et al. 2008, Spencer et al. 2008].

KEY WORDS: cattle / cloning / endometrium / estrus / infertility / pregnancy / transcriptome

*Supported by the Deutsche Forschungsgemeinschaft (FOR 478 “Mechanisms of embryo-maternal communication”) and by the BMBF (Projects “Fertilink”, “Remedy”, and “Compendium”).

**Corresponding author: ewolf@lmb.uni-muenchen.de
Transcriptome changes in bovine endometrium during the estrous cycle

Although the basic principles of hormonal regulations in the endometrium during the estrous cycle are known, the detailed molecular mechanisms are not well understood. To get a first insight into such mechanisms two analyses of transcriptome changes in bovine intercaruncular endometrium during the estrous cycle were performed. In the first study late estrus (day 0, low progesterone) and diestrus phase (day 12, high progesterone) were compared using a combination of subtracted cDNA libraries and cDNA array hybridization [Bauersachs et al. 2005]. This study revealed 133 genes showing at least a two-fold change of their mRNA abundance, 65 with higher levels at estrus and 68 with higher expression at diestrus. In the second study [Mitko et al. 2008] endometrium samples were analyzed derived from several stages of the estrous cycle: estrus (day 0), metestrus (day 3.5), diestrus (day 12), late diestrus (slaughtered at day 18, high serum progesterone levels), and preestrus (slaughtered at day 18, low serum progesterone levels). For the generation of mRNA expression profiles a bovine oviduct and endometrium (BOE) array [Bauersachs et al. 2007] was used, which was developed based on a series of differential gene expression studies in endometrium (different stages of the estrous cycle, day 15 and day 18 pregnant vs. non-pregnant) [Bauersachs et al. 2005, Bauersachs et al. 2006, Klein et al. 2006] and in oviduct epithelial cells (different stages of the estrous cycle) [Bauersachs et al. 2003, Bauersachs et al. 2004]. Analysis of expression data revealed 269 genes with significant changes in their transcript levels during the estrous cycle in distinct temporal patterns. Two major types of expression profiles were observed that showed highest mRNA levels during the estrous phase or highest levels during the luteal phase, respectively. A minor group of genes exhibited highest mRNA levels on day 3.5. The number of differentially expressed genes during the estrous cycle was comparable to those found in similar studies in mouse, Rhesus monkey and human, which have been done with high-density cDNA or oligonucleotide arrays, respectively. Gene Ontology classification of the genes with known function characterized the estrus time by elevated expression of genes related to focal adhesion formation, cell motility, cytoskeleton, extracellular matrix (ECM), ECM remodeling, and cell growth.

Transcriptome changes in the endometrium during the pre-implantation phase

Transcriptome analyses of bovine endometrium comparing samples recovered from day 18 pregnant animals and corresponding non-pregnant controls were performed using two different experimental models. In the first model monozygotic twin cows were used, where one twin received two in vitro-produced embryos and the corresponding twin a sham transfer at day 7 of the estrous cycle [Klein et al. 2006]. Endometrial tissue samples were recovered on day 18 of pregnancy and the estrous cycle, respectively. Using this genetically defined model system, 87 different genes
were identified as upregulated in pregnant animals. Almost half of these genes have been described as classical type I interferon-induced genes, i.e., is probably induced by interferon tau (IFNT), the embryonic pregnancy recognition signal in ruminants. In the second model pregnancy was obtained by artificial insemination of heifers [Bauersachs et al. 2006]. Control animals received a sham insemination (sperm cells were removed by centrifugation). Endometrial tissue samples were recovered on day 18 of pregnancy and the estrous cycle, respectively. In contrast to the first model, the control animals showed low serum progesterone levels due to a shortened estrous cycle. Thus the differential expression of the identified genes could be based on embryonic signals and/or hormonal differences. In this study 179 differentially expressed genes were found, 109 with higher and 70 with lower mRNA abundance in pregnant animals. Among the mRNAs with higher abundance in pregnant animals at least 41 are already described as induced by interferons. In addition a number of mRNAs encoding proteins that are involved in specific protein modification processes, such as ISG15ylation and ADP-ribosylation, were identified as upregulated in endometrium samples of pregnant animals. The ISG15 ubiquitin-like modifier has been hypothesized to be a critical component of the microenvironment at the uterine placental interface during the progressive events of conceptus development, implantation, and placentation. ISG15 is an ubiquitin-like protein that is conjugated to a number of target proteins thereby regulating their functions. Another protein modification process, ADP-ribosylation, was represented by three up-regulated mRNAs coding for members of the poly(ADP-ribose) polymerase (PARP) superfamily (PARP9, PARP10, and PARP12). A number of mRNAs coding for a variety of transcription factors was also identified as upregulated in endometrium of pregnant animals. For instance, NR2F2 (COUP-TFII), a nuclear orphan receptor, has been shown to repress the human oxytocin gene promoter in uterine epithelial cells. In mice, NR2F2 has been shown to regulate stromal cell differentiation of estrogen activity required for establishing a receptive uterus. A conditional knockout of Nr2f2 in the ovary and the uterus led to severely impaired placental formation that results in miscarriage, whereas haploinsufficiency of Nr2f2 in the mouse results in decreased progesterone synthesis in the CL leading to reduced ability of the endometrium to support pregnancy.

The modulation of the maternal immune system is essential to prevent rejection of the conceptus, which represents a semi-allograft. The transcriptome analyses of pregnant endometrium revealed an upregulation of several immune-related genes. Four of them are involved in the complement system (classical pathway), namely complement component genes C1S, C1R, C4, and the C1 inhibitor SERPING1. In situ hybridization experiments revealed specific expression of C1S, C1R, and SERPING1 mRNA mainly in luminal and glandular epithelial cells, but also weak expression in stromal cells. The simultaneous upregulation of SERPING1, encoding a protein known as C1 inhibitor, in the luminal and glandular epithelial cells could be a mechanism to protect the embryo against an attack by the complement system. UTMP (SERPINA 14) is another gene that may play a role in the modulation of the maternal immune system.
immune system. For ovine UTMP protein inhibition of NK-like activity was shown and a role in protecting the conceptus from maternal cytotoxic lymphocytes has been suggested.

The process of embryo attachment is essentially dependent on cell adhesion processes. A functional classification of the up-regulated genes revealed a number of genes, which may be involved in the process of cell adhesion between embryonic trophoblast cells and cells of the luminal epithelium: AGRN, CD81, LGALS3BP, and LGALS9. In situ hybridization for AGRN, LGALS3BP, and LGALS9 showed strong staining in the luminal epithelium in pregnant animals.

In addition, a number of candidate genes for endometrium remodelling were found, such as MEP1B, legumain (LGMN), MMP19, TIMP2, transglutaminase 2 (TGM2), met protooncogene (hepatocyte growth factor receptor, MET), and epithelial stromal interaction 1 (EPSTI1). The asparaginyl-specific cysteine proteinase LGMN may play a role in regulation of ECM remodeling as it has been shown to activate MMP2 in vitro and in cultured cells. With MMP19 and TIMP2 two genes coding for components of the matrix metalloproteinase system were identified. In addition to the upregulation of TIMP2 mRNA at day 18 of pregnancy in bovine endometrium, TIMP2 mRNA levels are higher at day 12 compared to day 0 of the estrous cycle. These findings suggest an important role of MMP19 and TIMP2, the inhibitor of MMP2, for the regulation of attachment of the conceptus. Furthermore, mRNA of TIMP1, coding for a tissue inhibitor of metalloproteinases, was decreased in endometrium of day 18 pregnant animals compared to the control group.

A summary of the processes probably important in endometrium of the peri-implantation period is given in Figure 1.

E. Wolf, S. Bauersachs

Fig. 1. Transcriptome changes of the bovine endometrium during the peri-implantation period [from Bauersachs et al. 2006].
Evaluation of endometrium transcriptome changes in response to embryos derived by assisted reproduction techniques (ART)

Assisted reproduction techniques (ART) are becoming increasingly important in human reproductive medicine and in animal breeding and biotechnology as well. Although in vitro fertilization 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 by ART as compared to natural reproduction.

Somatic cell nuclear transfer (SCNT) cloning, which has been successful in a number of species, is particularly critical with respect to epigenetic abnormalities of the resulting embryos, fetuses and offspring [reviewed in Shi et al. 2003]. For instance, we observed premature DNA methylation in a significant proportion of bovine somatic cell nuclear transfer embryos [Dean et al. 2001, Santos et al. 2003]. DNA hypermethylation was observed in some tissues of cloned bovine fetuses, but – to a lesser extent – also in fetuses derived from in vitro-produced embryos [Hiendleder et al. 2004b, Hiendleder et al. 2006]. However, it is largely unclear whether and how epigenetic changes cause developmental abnormalities and abortions of cloned embryos or fetuses. 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 [reviewed in Bauersachs et al. 2009].

Furthermore, we observed transplacental leakage of maternal cells into the circulation of fetuses derived by SCNT, but not in IVF-derived fetuses [Hiendleder et al. 2004a]. These findings raise the question, how and when these changes of placental functionality are induced. The fact that genes which may be involved in placenta formation were found to be abnormally expressed in SCNT embryos invited the concept that placental abnormalities may have their origin in abnormal embryo-maternal communication already in the preimplantation period. To clarify this hypothesis, we initiated a study evaluating transcriptome profiles of endometrium samples in response to SCNT embryos vs. embryos derived by in vitro-fertilization (IVF). Importantly, several different nuclear donor cell lines were used for SCNT to have a similar genetic variation in the SCNT and the IVF groups, excluding specific effects of a particular embryonic genotype as cause for transcriptome differences in the corresponding endometrium samples. SCNT embryos and IVF embryos were cultured under identical conditions to the blastocyst stage (Day 8) and transferred to recipients, which were slaughtered at day 18 of pregnancy.

The mRNA profiles of endometrium samples were obtained using a custom cDNA microarray enriched for transcripts differentially expressed in the endometrium and/or oviduct epithelium during the estrous cycle and/or early pregnancy [BOE array; Bauersachs et al. 2007]. Overall, variation of mRNA profiles was greater in the SCNT group than in the IVF group. Furthermore, 58 transcripts were differentially abundant between endometria from SCNT vs. IVF pregnancies (Fig. 2). Prominent examples are
NR2F2 (orphan nuclear receptor COUP-TFII) and GJA1 (connexin 43), both known to play important roles in uterine receptivity and conceptus placentation. These findings suggest that placental failure in bovine clone pregnancies originate from abnormal embryo-maternal communication that develops during the peri-implantation period. Endometrium transcriptome profiles may serve as a novel tool to evaluate SCNT embryos for their ability to establish pregnancy and develop a functional placenta [Bauersachs et al. 2009].

Conclusions and Perspectives

Transcriptome profiling is a first step for identifying molecular mechanisms underlying the functional changes in the endometrium during the estrous cycle and during pregnancy. Our previous studies identified transcriptome profiles which are characteristic for pregnant vs. non-pregnant animals or for different stages of the estrous cycle. 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. Our first study of endometrium proteome changes in pregnant animals (day 18 = pre-attachment period) revealed four proteins with higher abundance in pregnant endometrium, which have
previously not been known to be regulated in this period in bovine [Berendt et al. 2005]. Novel proteomics techniques, such as highly sensitive saturation labeling [Berendt et al. 2009], will further increase the analytical depth of proteome studies and facilitate analyses of microdissected tissues.

In addition to questions related to basic research, endometrial transcriptome profiles may have important implications for the cattle breeding industry. At the moment improvement of fertility by genetic selection is hampered by the low heritability of currently recorded fertility traits, e.g., the non-return rate 90 days after artificial insemination. Future studies will clarify whether transcriptome profiles of endometrium biopsies taken at a specific stage of the estrous 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 realizing the concept of genomic selection for fertility.

REFERENCES


Genomika funkcjonalna w biologii rozrodu
i biotechnologii – praca przeglądowa

**Streszczenie**
