

# Combined analysis of transcriptome studies of bovine endometrium during the preimplantation phase and comparison to results from ovine and porcine preimplantation endometrium

Stefan Bauersachs

*ETH Zurich, Animal Physiology, Institute of Agricultural Sciences, Department of Environmental Systems Science, Universitaetstrasse 2 / LFW B 58.1, 8092 Zurich, Switzerland*

## Summary

Transcriptome studies of the bovine endometrium during early pregnancy and during the estrous cycle have been performed using DNA microarrays and RNA sequencing to identify genes associated with establishment and maintenance of pregnancy. The results of these studies are reviewed and the identified differentially expressed genes (DEG) are compared to define the overlaps between studies and technical platforms. Good overlaps were found for upregulated genes between different days of the preimplantation phase and between microarrays and RNA-Seq. In contrast, overlaps for downregulated genes were clearly lower between days but also between platforms. Possible causes are discussed. All DEG of the analyzed studies were summarized and a combined functional annotation analysis was performed. Gene expression of selected genes found as important for maternal recognition of pregnancy and implantation in sheep was compared between bovine and ovine endometrium. Furthermore, DEG in bovine endometrium were compared to DEG found in porcine endometrium on Day 14 of pregnancy, revealing significant similarities in differential endometrial gene expression in cattle and pigs.

## Introduction

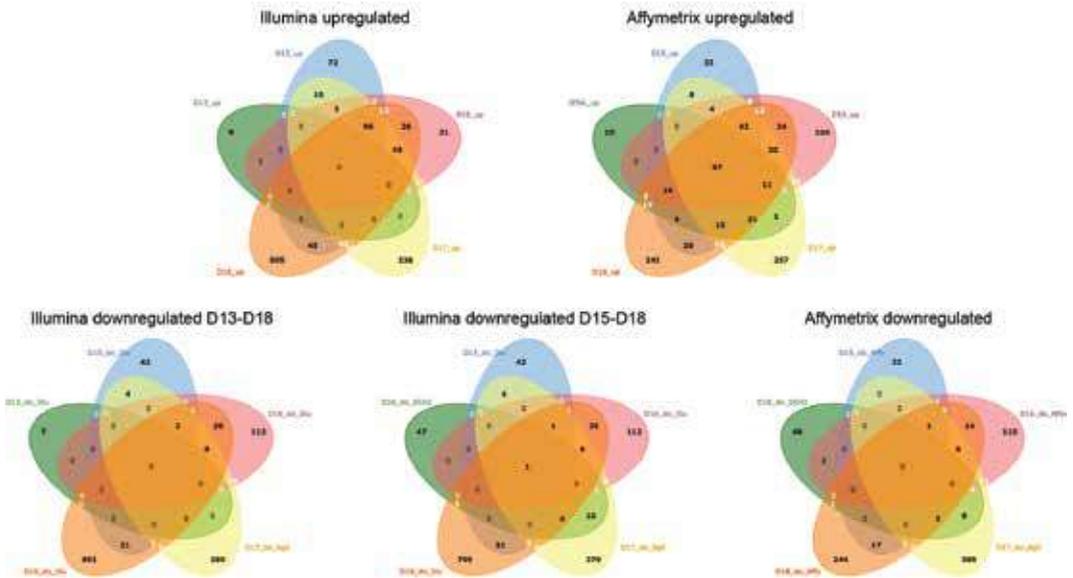
The successful development of the bovine conceptus during the preimplantation phase is strongly dependent on the maternal environment, which is mainly controlled by the ovarian steroid hormones. The most important steroid hormone for uterine receptivity is progesterone (P4) (Spencer & Bazer 2002, Bazer *et al.* 2008). The importance of P4 for preimplantation conceptus growth and development in cattle has been shown in several studies with elevated P4 in the post-conception period (Clemente *et al.* 2009, Forde *et al.* 2009), exogenous supplementation of P4 and induction of low circulating P4 (Forde *et al.* 2011a), P4 treatment to compensate asynchronous embryo transfer (ET) (Geisert *et al.* 1991), and other studies using asynchronous ET as a model for the study of endometrial receptivity (Ledgard *et al.* 2012). On the other hand, in ruminants the maintenance of the production of progesterone is dependent on the embryonic pregnancy recognition signal interferon tau (IFNT), which prevents luteolysis (Thatcher *et al.* 1989) and modifies endometrial gene expression. Since expression of type I and/or type II interferons (IFNs) has been found in many mammals during the pre- and periimplantation phase, modulation of endometrial gene expression by these cytokines probably plays a significant role not only for pregnancy recognition in ruminants but for embryo implantation in general (Bazer

*et al.* 2009). The complex interplay of P4, IFNT, and other conceptus and endometrium derived factors, such as prostaglandins, is the basis for proper conceptus development in preparation for implantation and placentation (Dorniak *et al.* 2013).

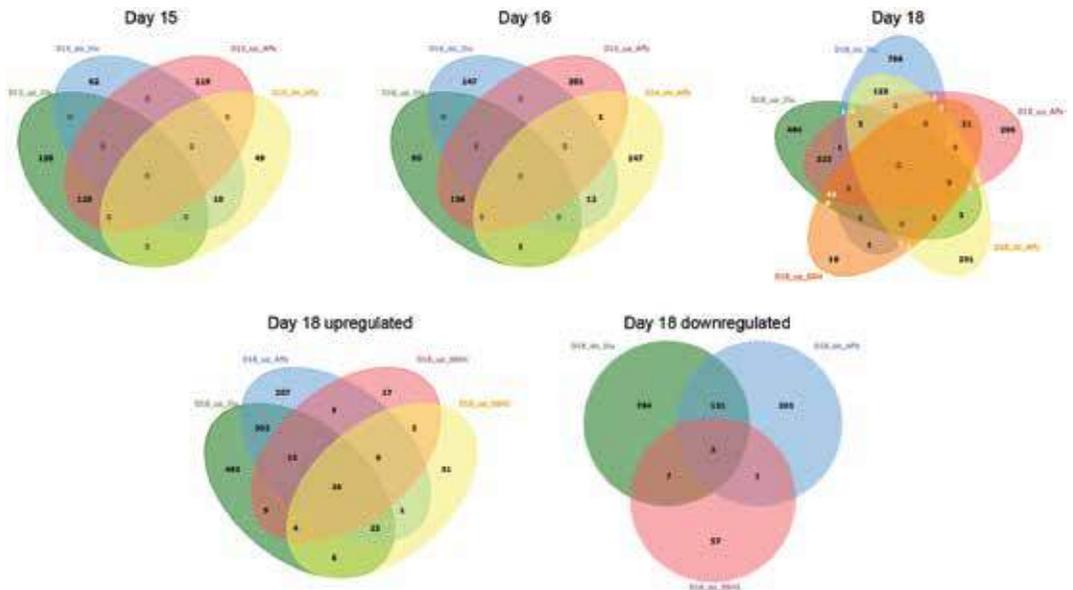
In the last decade, many holistic studies of gene expression in the bovine endometrium have been performed for different stages of the estrous cycle and the pre- and periimplantation period (reviewed in Forde & Lonergan 2012, Ulbrich *et al.* 2013), showing very complex gene expression changes underlying the complex physiological changes in this tissue. In a previous study, we compared the results of a number of transcriptome studies performed on different days of early pregnancy and found good agreement even though different technical platforms and different gene annotations were used and the studies were performed in different laboratories (Bauersachs *et al.* 2012). In this review the most relevant endometrial transcriptome studies performed to date are summarized and the overlaps between studies and technological platforms are characterized. In addition, results are compared to findings in ovine and porcine endometrium during the preimplantation phase.

### **Comparison of differentially expressed genes identified in transcriptome studies in bovine endometrium during the preimplantation phase**

A considerable number of transcriptome studies using DNA microarrays and more recently RNA sequencing (RNA-Seq) have been performed for different days of the preimplantation phase in bovine endometrium ranging from Day 13 to Day 18 of pregnancy. Although these studies have been performed from different groups, by the use of different technical platforms, and with different approaches for data analysis, a consistent overlap was found for the studies on Days 15, 16, 17 and 18 of pregnancy by the use of Gene Set Enrichment Analysis (GSEA) (Bauersachs *et al.* 2012). The results of a new comparison of the identified differentially expressed genes (DEG) are shown in Fig. 1. The comparison was based on data from the very first transcriptome studies of bovine endometrium using a combination of subtracted cDNA libraries and cDNA microarrays (Bauersachs *et al.* 2006, Klein *et al.* 2006), studies performed with the Affymetrix Bovine Genome Array (Forde *et al.* 2011b, Bauersachs *et al.* 2012), one study using Agilent microarrays (Walker *et al.* 2010), and in addition, studies implementing RNA sequencing (Bauersachs & Wolf 2012, Forde *et al.* 2012). Although the lists of DEG provided with the individual publications were very heterogeneous regarding gene or transcript identifiers, if provided at all, gene annotation was updated to the same state and to get comparable gene identifiers (gene symbols). Altogether, more than 3300 genes were found as differentially expressed (Supplemental Table 1) on the studied days of pregnancy. The comparison of the lists of DEG did not show an overlap of the genes identified on Day 13 of pregnancy (Forde *et al.* 2012) with the DEG obtained from all later time points, neither for upregulated nor for downregulated genes (Fig. 1, top and bottom left). In concordance with the results from GSEA, relatively good overlaps were found for the upregulated genes, e.g., 288 genes found as upregulated on Day 15 (Illumina data set, Fig. 1, top left) and on at least one of the following time points and only 72 genes were found only on Day 15. Similarly for Day 16 (Forde *et al.* 2012), 198 genes were found as upregulated on Day 16 and on at least one other time point in contrast to only 31 found solely for Day 16. For the 540 genes found as upregulated on Day 17 of pregnancy (Walker *et al.* 2010), 304 genes were also identified in one or more of the other studies. Similar overlaps were found for the comparison of the DEG derived from the microarray studies (Fig. 1, top right). In contrast, the overlap for the downregulated genes was much lower, particularly for those obtained for Days 16 and 17 of the microarray studies (Walker *et al.* 2010, Forde *et al.* 2011b) (Fig.1, bottom right).



**Fig. 1.** Overlap of differentially expressed genes found in transcriptome studies for different days of the preimplantation phase. Venn diagrams were produced based on gene symbols of the reannotated gene lists using the web tool jquery.venny. D: Day; IFNA: interferon alpha; Agil: Agilent; SSH: Suppression Subtractive Hybridization; SSH2: Bauersachs *et al.* 2006.



**Fig. 2.** Overlap of differentially expressed genes for different technological platforms. Venn diagrams were produced based on gene symbols of the reannotated gene lists using the tool jquery.venny. D: Day; SSH: Suppression Subtractive Hybridization; SSH1: Klein *et al.* 2006; SSH2: Bauersachs *et al.* 2006.

The comparison of RNA-Seq and microarrays showed clear lower overlap for downregulated genes. Fig. 2 shows a comparison of the different technology platforms for the days where studies with different platforms have been performed. Also between platforms, the overlap

of DEG was clearly lower for the downregulated genes. A comparison of the median of the corrected P-values for downregulated and upregulated genes revealed that P-values were better for upregulated genes compared to the downregulated genes for all studies (Table 1). In general, the main limitations for such comparisons are probably differences in statistical analysis of gene expression data, limited numbers of investigated biological replicates, and gene annotation, which is very dynamic, i.e., changes with time. For example, in the Agilent microarray study of Day 17 of pregnancy (Walker *et al.* 2010), a false discovery rate (FDR) of 5% was used. In consideration of the number of DEG found in this study, about 60 genes are likely to be false positives (5% of 1189).

**Table 1.** Median P-values for downregulated and upregulated genes

Study	Downregulated	Upregulated	Replicates
Day 15 Illumina	0.00035	0.00012	3
Day 15 Affymetrix	0.0334	0.0140	3
Day 16 Illumina	0.0003401	0.0000006	5
Day 16 Affymetrix	0.018	0.002	5
Day 17 Agilent	0.0080	0.0037	10,12*
Day 18 Illumina	0.00057	0.00014	4
Day 18 Affymetrix	0.0101	0.0046	4

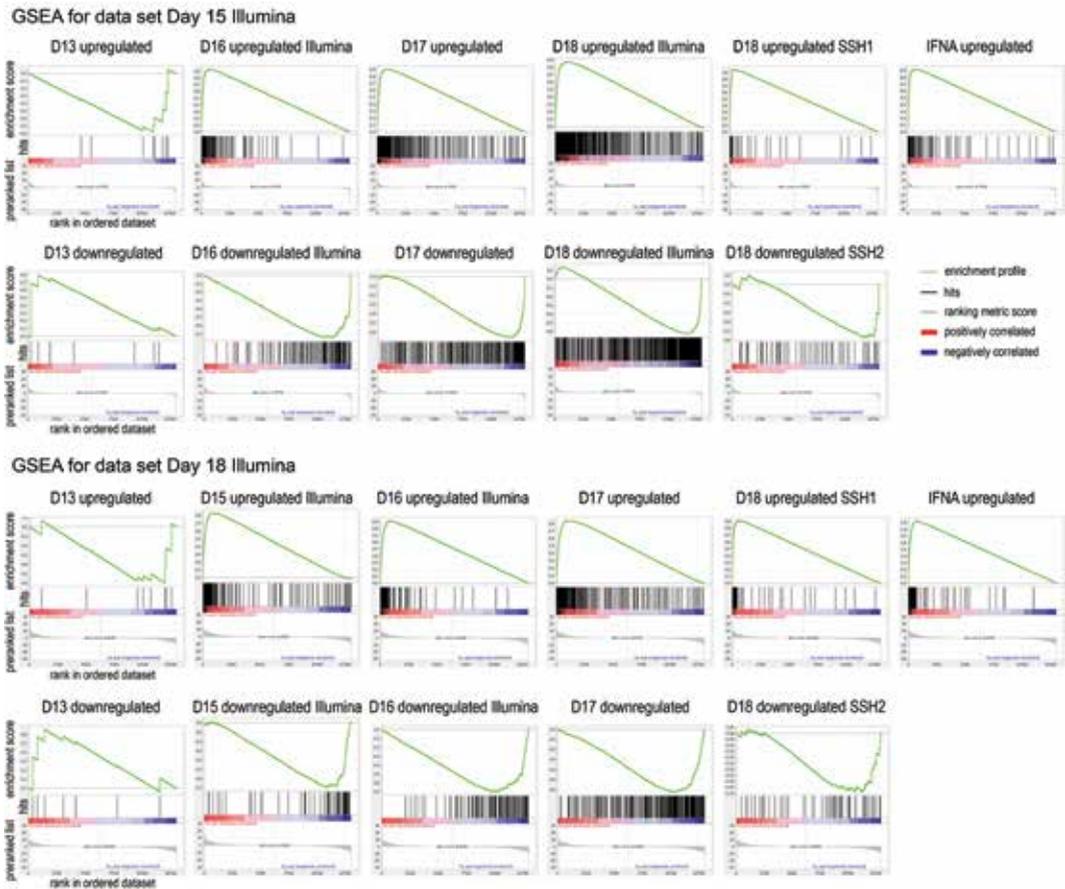
\*10 cyclic and 12 pregnant cows

However, the results of GSEA show that a clear enrichment towards the expected end of the ranked data sets for Days 15 and 18 of pregnancy is also present for the downregulated genes (Fig. 3). This indicates that expression differences and corrected P-values of a considerable part of the “nonoverlapping” genes, i.e., DEG found in only one study, are near by but below the thresholds set in the individual studies. In addition, it is also likely that some of the DEG show differential expression only during a distinct and short phase of early pregnancy and therefore were found only once.

Despite of the limitations of the technical platforms for transcriptome analyses, limited numbers of biological replicates in most studies and different strategies and thresholds for statistical analysis, the performed studies revealed first changes in the endometrial transcriptome on Day 13 of pregnancy (Forde *et al.* 2012), substantial differences on Day 15 (Bauersachs *et al.* 2012) and increasing numbers of DEG up to Day 18 of pregnancy. In our own studies, we analyzed also Day 12 of pregnancy but did not find significant changes in whole endometrial tissue samples (S Bauersachs, H D Reichenbach, H Blum & E Wolf 2008, unpublished observations). Particularly for Days 15 and 16, more upregulated than downregulated genes were found, probably due to the effects of IFNT resulting predominantly in activating responses.

### Functional groups overrepresented in the identified differentially expressed genes

A summarization of the DEG found in the analyzed studies is shown in Supplemental Table 1 with the frequency with which the genes have been found as well as the corresponding studies. For some gene families, similar regulation for all or almost all members of the family was observed. Some members of such gene families were only identified in one or two studies. Details are shown in Table 2 for one example, the homeobox A (*HOXA*), *HOXB* and *HOXC* gene



**Fig. 3.** Comparison of the RNA sequencing data sets for Days 15 and 18 of pregnancy with the sets of differentially expressed genes of the other studies. Gene set enrichment analysis (Subramanian *et al.* 2007) was performed for preranked (based on a score calculated from fold change and P-value) lists of detectable genes in the RNA-Seq analysis for Days 15 and 18 of pregnancy, respectively, in comparison to the sets of differentially expressed genes of the other studies. D: Day; IFNA: interferon alpha; SSH: Suppression Subtractive Hybridization; SSH1: Klein *et al.* 2006; SSH2: Bauersachs *et al.* 2006.

families. All members of these HOX families show lower expression in pregnant endometrium from Day 15 through Day 18. These homeobox transcription factors have been shown to play a role in regulation of cell differentiation of various cell types such as hematopoietic cells (Magli *et al.* 1997), endothelial cells (Minten *et al.* 2013), and normal epithelial and tumor cells (Venhoranta *et al.* 2013). Downregulation of transcription was observed for the complete *HOXB* gene cluster, but also for 4 members of the *HOXA* cluster and two genes of the *HOXC* family (Supplemental Table 1). So far, only upregulation of *HOXA10* and *HOXA11* has been shown in murine and human endometrium during implantation (Eun Kwon & Taylor 2004). However, the downregulation of HOX genes could be associated with a differentiation status of endometrial cells needed for the preparation for conceptus implantation. A role of *HOXB* genes in context of morphogenesis has been found, for example, also in the lung of the mouse

**Table 2.** Summary of differentially expressed HOX genes

Gene symbol	Gene description	Bta Gene ID	Hsa Gene ID	Frequency	Study(ies)
<i>HOXA3</i>	homeobox A3	509991	3200	1	D18 down Illu
<i>HOXA4</i>	homeobox A4	538865	3201	1	D16 down Affy
<i>HOXA6</i>	homeobox A6	100848736	3203	1	D18 down Illu
<i>HOXA7</i>	homeobox A7	615851	3204	1	D15 down Illu
<i>HOXB2</i>	homeobox B2	616129	3212	4	D16 down Illu,D17 down,D18 down Affy,D18 down Illu
<i>HOXB3</i>	homeobox B3	618214	3213	1	D18 down Illu
<i>HOXB4</i>	homeobox B4	768240	3214	2	D16 down Illu,D18 down Affy
<i>HOXB5</i>	homeobox B5	ENSBTAG00000045835	3215	2	D15 down Affy,D18 down Affy
<i>HOXB6</i>	homeobox B6	100337380	3216	4	D15 down Affy,D15 down Illu,D18 down Affy,D18 down Illu
<i>HOXB7</i>	homeobox B7	281820	3217	2	D16 down Illu,D18 down Affy
<i>HOXB8</i>	homeobox B8	785855	3218	3	D15 down Affy,D16 down Affy,D18 down Affy
<i>HOXC10</i>	homeobox C10	100847795	3226	1	D17 down
<i>HOXC6</i>	homeobox C6	317660	3223	1	D17 down

fetus via regulation of ECM expression of tenascin C (TNC) and modulation of FGF10 spatial expression (Volpe *et al.* 2007). In concordance with this finding, DAVID Functional Annotation Clustering revealed overrepresentation of functional terms related to cell differentiation such as “tissue morphogenesis” and “epithelium development” (Supplemental Table 2a).

Members of the WNT signaling pathway were also assigned to these or similar categories and were likewise predominantly downregulated (frizzled family receptors [*FZD3*, *FZD4*, *FZD5*, *FZD10*], wingless-type MMTV integration site family members [*WNT5A*, *WNT6*, *WNT9A*], WNT1 inducible signaling pathway protein genes 1 and 2 [*WISP1*, *WISP2*]). Downregulation of WNT signaling by progesterone has been shown in ovine and human endometrium (Satterfield *et al.* 2008, Wang *et al.* 2009).

Another group of genes, which were primarily downregulated, were collagen genes (20 genes, for details see Supplemental Table 1), mainly at later time points of the preimplantation phase. This could be due to preparation of the endometrium for the next estrus during the follicular phase in the cyclic control animals. A second group of genes probably related to extracellular matrix (ECM) remodeling was the ADAM metallopeptidase domain and the ADAM metallopeptidase with thrombospondin type 1 motif gene family, which was also mainly downregulated. Overrepresentation of ECM-related functional categories for the genes downregulated in pregnant endometrium was confirmed with the results from DAVID Functional Annotation Clustering (Supplemental Table 2a).

The upregulated genes were, in addition to immune response-related genes, enriched for genes coding for mitochondrial proteins such as mitochondrial ribosomal proteins, ATP synthases, NADH dehydrogenase subunits, and a number of other mitochondrial enzymes. However, most of the overrepresented functional categories were related to various immune response functions and processes (Supplemental Table 2b).

### Comparison to gene expression in ovine endometrium for genes associated with maternal recognition of pregnancy and conceptus implantation

The model of maternal recognition of pregnancy (MRP) in ruminants, mainly established based on studies in sheep, comprises upregulation of the transcriptional repressor interferon regulatory factor 2 (*IRF2*) in the luminal epithelium (LE). *IRF2* suppresses the upregulation of *ESR1* and *OXTR* eventually leading to the prevention of the release of PGF2a (Spencer *et al.* 2007). In bovine endometrium, *IRF1* and *IRF3* to *IRF9*, but not *IRF2*, were found as upregulated in pregnant animals. *IRF2* expression was detected in the transcriptome studies but with similar levels in cyclic and pregnant endometrium. It is possible that upregulation of *IRF2* also occurs in bovine endometrium, but specifically in the LE and thus not detectable in the analysis of whole endometrial tissue samples. Like in sheep, *ESR1* and *OXTR* are also repressed in bovine endometrium.

In sheep, a number of genes have been shown to be important for conceptus attachment and implantation, such as secreted phosphoprotein 1 (osteopontin, *SPP1*), galectin 15 (*LGALS15*), and glycosylation-dependent cell adhesion molecule 1 (*GLYCAM1*) (Spencer *et al.* 2004). *SPP1* is also upregulated in pregnant bovine endometrium (Supplemental Table 1; Days 15, 17 and 18). The putative ortholog of ovine *LGALS15* is not expressed in bovine endometrium. However, other members of the galectin gene family are expressed and *LGALS7* and *LGALS9* have been found as upregulated in pregnant endometrium (*LGALS9* from Day 15 to 18). Localization of *LGALS9* mRNA expression in bovine endometrium has been shown in luminal epithelium (Bauersachs *et al.* 2006) similar to the localization found in human endometrium (Popovici *et al.* 2005). These findings indicate that other galectins may have a similar function in conceptus attachment as *LGALS15* in ovine endometrium. *GLYCAM1* was not found to be expressed in bovine endometrium consistent with data from the UniGene database where 215 expressed sequence tags (ESTs) are listed, which were almost exclusively found in the mammary gland with only 3 ESTs from the liver. Like the human *LGALS15* ortholog, *GLYCAM1* is a pseudogene in humans. The expression of another protein relevant for the process of conceptus attachment, mucin 1 (*MUC1*), is downregulated in the LE in ovine endometrium during the preimplantation phase (Johnson *et al.* 2001). In bovine endometrium, *MUC1* was detected but not downregulated in pregnant endometrium. However, since the downregulation is specific for the LE this is probably hidden in the analysis of whole endometrial tissues.

Integrins have also been shown to be involved in the attachment of trophoblast and LE cells. A constitutive expression has been shown in ovine trophoblast and endometrial cells for the integrin subunits alpha (*ITGAV*, *ITGA4*, *ITGA5*) and beta (*ITGB1*, *ITGB3* and *ITGB5*) (Johnson *et al.* 2001). In contrast, a number of integrin genes are differentially expressed in bovine endometrium, at least at the mRNA level. *ITGA3*, *ITGA4*, *ITGA10*, *ITGAV*, and *ITGB6* were found as upregulated and *ITGA7*, *ITGAE*, and *ITGB8* as downregulated (Supplemental Table 1). Some of these integrin genes show a similar regulation in porcine endometrium on Day 14 of pregnancy, namely *ITGA4*, *ITGAV*, and *ITGB6* (Samborski *et al.* 2013a). Overall, this comparison indicates that there are similarities but also distinct differences even between closely related species such as cattle and sheep.

### Comparison to results from porcine endometrium on Day 14 of pregnancy

Since there are some similarities between cattle and pigs during the preimplantation phase, we recently performed a comparison of results from Day 18 of pregnancy in bovine endometrium (Bauersachs *et al.* 2012) to Day 14 of pregnancy in porcine endometrium (Samborski *et al.*

2013a). This comparison of the bovine Day 18 Affymetrix microarray data set and the porcine Day 14 RNA-Seq data set revealed a total overlap of 239 genes out of 1512 porcine genes, where 34 genes were found as downregulated in both studies, 180 were upregulated in both studies, and 25 genes showed contrary changes (Samborski *et al.* 2013a). Using all DEG of the bovine studies for Days 15, 16, 17 and 18 (omitting genes upregulated on one and downregulated on other days of pregnancy), the total overlap was 505 out of 1512 porcine genes (Supplemental Table 3). In both studies, 124 genes were found as downregulated and 292 as upregulated, respectively. Eighty-nine of the genes found in both species showed contrary changes. Both comparisons revealed many more overlapping genes than expected by chance. In addition, more than 80% of the overlapping genes showed similar regulation in both species further confirming the specificity of the observed overlap.

DAVID Functional Annotation Clustering for the genes upregulated in both species mainly revealed functional terms related to immune response as overrepresented (Supplemental Table 4a). In addition, genes involved in regulation of apoptosis were enriched in the upregulated genes. Overrepresented functional categories for the genes downregulated in both species were, for example, “extracellular matrix”, “calcium ion binding”, and “transcription factor activity” (*CREB3L4*, *ESR1*, *HOXA3*, *HOXB5*, *HOXB6*, *HOXB8*, *NR2F6*, *SPDEF*, *TCF7L1*, *ZNF202*) (Supplemental Table 4b). Interestingly, some members of the HOX family were also downregulated in porcine endometrium on Day 14 of pregnancy as well as on Day 12 of pregnancy (*HOXA3*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, *HOXB8*) (Samborski *et al.* 2013b). A number of overrepresented functional terms were also found for the genes with opposite regulation, such as “extracellular region”, “cell adhesion”, “cytoplasmic membrane-bounded vesicle”, “extracellular matrix”, and “cell motion” (Supplemental Table 4c).

### **Strategies and approaches to obtain deeper insights into processes related to establishment and maintenance of pregnancy**

All of the above-described studies have been performed using complete endometrial tissue samples. However, this tissue is highly complex and contains various cell types, such as luminal and glandular epithelial (GE) cells, stromal cells, endothelial cells and a variety of immune cells. This results in an average of gene expression over all endometrial cells, which could hide cell-specific changes or neutralize opposite changes in different cell types. Incorrect conclusions with respect to interpretation of changes in context of molecular pathways when two “interacting” genes are actually not expressed in the same cell type could also occur. Results of other groups and our own results indicate differences in responses to hormonal control and signals of the embryo in specific endometrial compartments. For example, a comparison of two microarray studies of similar stages of cyclic endometrium (Day 7 vs. Day 14) using the same Affymetrix bovine microarray (Salilew-Wondim *et al.* 2010, Forde *et al.* 2011a) revealed a very small overlap, not higher than expected by chance. This finding strongly indicates cell type-specific gene expression changes in the bovine endometrium during the estrous cycle since different sampling techniques were used in these studies, whole endometrial tissue samples in Forde *et al.* (2011a) and cytobrush samples in Salilew-Wondim *et al.* (2010). Cytobrush samples mainly contain cells of the luminal surface, i.e. LE and superficial GE. A second example is a study of equine endometrial biopsy samples where one sample was found to have a strong deviation in the percentage of LE cells which resulted in distinct differences in gene expression (Merkl *et al.* 2010). The only way to overcome this issue will be the separate analysis of endometrial cell types by the use of laser capture microdissection for the collection of samples from distinct

endometrial compartments. Although the separate analysis of different endometrial cell types would considerably increase the complexity of transcriptome studies, it should be feasible using most current RNA-Seq technologies.

Additional approaches to identify new genes or pathways important for endometrial functions in context of establishment and maintenance of pregnancy could be the analysis of endometrial transcriptomes across different mammalian species (Bauersachs & Wolf 2012) and an extension of transcriptomics data with proteomics, e.g., the systematic analysis of uterine secretions during the preimplantation phase (Forde *et al.* 2014).

## Conclusions

The comparison of transcriptome studies in bovine endometrium during the preimplantation phase showed consistent gene expression changes, particularly for the genes upregulated in pregnant endometrium. This is in contrast to microarray studies performed for human endometrium (Riesewijk *et al.* 2003). The overlap between studies was lower for downregulated genes, which could be due to different reasons, e.g., higher variation of gene expression values obtained for these genes in whole endometrial tissue samples, a temporal regulation of these genes, and hormonal differences in the cyclic control animals, particularly for the later time points. The combined analysis of the summarized studies provides a more complete picture of gene expression changes during the preimplantation phase and, in addition, for the comparison to other mammals as shown in a comparison to porcine endometrium.

## Supplemental data

Supplemental data can be downloaded via: <https://polybox.ethz.ch/public.php?service=files&t=1a46ddd3942d7ecd93481fd9a6bb4439>

## Acknowledgements

The generation of data analyzed in this study was supported by the German Ministry for Education and Research (BMBF, FUGATO-plus, COMPENDIUM; FUGATO Fertalink) and the German Research Foundation (DFG FOR478). The author is part of the European Union COST action FA1201 Epiconcept.

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