



Review Article

Transcriptional profiling to address molecular determinants of endometrial receptivity – Lessons from studies in livestock species

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ARTICLE INFO

Article history:

Available online 21 November 2012

Communicated by Michael W. Pfaffl

Keywords:

Transcriptomics
Endometrium
Pregnancy
Livestock
Biomarker

ABSTRACT

The development of a fertilized oocyte into a differentiated multi-cellular organism is a major challenge with regard to the orchestration of the expression of the mammalian genome. Highly complex networks of genes are temporally and spatially regulated during cellular differentiation to generate specific cell types. Embryonic development is critically influenced by external impacts in the female reproductive tract. A most critical phase of pregnancy in mammals is the pre- and peri-implantation period, during which the uterine environment plays a crucial role in supporting the development of the conceptus. The analytical description of the transcriptome, proteome and metabolome of the embryo-maternal interface is a prerequisite for the understanding of the complex regulatory processes taking place during this time. This review lines out potentials and limitations of different approaches to unravel the determinants of endometrial receptivity in cattle, the pig and the horse. Suitable *in vivo* and *in vitro* models, which have been used to elucidate factors participating in the embryo-maternal dialog are discussed. Taken together, transcriptome analyses and specified selective candidate gene driven approaches contribute to the understanding of endometrial function. The endometrium as sensor and driver of fertility may indicate the qualitative and quantitative nature of signaling molecules sent by the early embryo and in turn, accordingly impact on embryonic development.

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1. Endometrial function in livestock species

Remodeling and differentiation processes in the female reproductive system during the sexual cycle and pregnancy provide a perfectly synchronized environment for final maturation of gametes, fertilization, embryonic development, and for the establishment of pregnancy. Hormones have a major impact on the physiological status of the endometrium [1]. At the site of ovulation, the functional corpus luteum develops and produces progesterone (P₄) to sustain the secretory property of the endometrium. The priming action of P₄ on endometrial function is essential for its ability to obtain a receptive state. Progesterone exposure leads to an obligatory loss of progesterone receptor (PGR) from the luminal (LE) and glandular epithelium (GE) of the endometrium [2]. The

Abbreviations: AA, amino acid; GE, glandular epithelium; IFNT, interferon- τ ; IRF, interferon regulatory factor; ISG, interferon-stimulated gene; LE, luminal epithelium; MRP, maternal recognition of pregnancy; P₄, progesterone; PCA, principal component analysis; PG, prostaglandin; PGR, progesterone receptor; RIN, RNA integrity values; SCNT, somatic cell nuclear transfer; SDV, screen tape degradation values; SERPINA14, uterine milk protein.

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loss of epithelial PGR is accompanied by sustained PGR-positive stromal cells, which in turn produce progesterone-like factors (i.e., growth factors), that may act in a paracrine mode on both the epithelial cells and the trophoblast [3]. This allows continuous P₄-mediated endometrial secretion and creates an environment suitable for early embryonic development and implantation. The uterine fluid, also known as uterine milk or histotroph, comprises not only nutrients but also signaling molecules, including growth factors, cytokines, and hormones of both maternal and embryonic origin for bidirectional communication purposes [4].

The establishment of pregnancy is a critical phase. Besides a suitable maternal environment, the embryo needs to signal its presence and to intermit luteal regression [5]. In primates including women, invasive implantation commences six days after fertilization and the pregnancy recognition signal (i.e., human chorionic gonadotropin of trophoblast origin) is directly secreted into the maternal circulation. In contrast, ungulates, such as bovine, porcine and equine species, show a longer pre-attachment development and a non-invasive type of embryo implantation, where pregnancy recognition before implantation is of critical importance. Therefore, endometrial function is adapted to the prolonged period of supporting embryonic development by secretions into the uterine lumen (histotrophic nutrition).

The window of time for maternal recognition of pregnancy (MRP), the embryonic signaling molecules involved and their mode of action differ between species [6,7]. In ruminants, the major embryonic pregnancy recognition signal is interferon- τ (IFNT), a type I interferon. Following hatching, the embryo begins to express IFNT, and the concentration increases remarkably during conceptus elongation [8]. In addition to IFNT, other signals are secreted by the ruminant conceptus. These either directly target the corpus luteum (CL) to eventually enhance P_4 production, or act on the endometrium to promote blood flow and vascular supply [9] in order to enhance the immune-privileged state of the uterus [10] and to support embryonic growth and development [11]. Prostaglandins (PG) are among the factors which have been shown to be crucial for conceptus elongation *in vivo* [12]. Porcine concepti synthesize estrogens as antiluteolytic signal [13,14], which act locally on the endometrium. Estrogens can act in a direct luteotropic manner to stimulate P_4 secretion from the corpus luteum [15], but also indirectly to increase luteal luteinizing hormone receptor [16], to reduce peripheral $PGF_{2\alpha}$ [17] and to modify endometrial PG synthesis (reviewed in [18]). Furthermore, the production of INF γ and IFN δ [19] has as well been demonstrated in the pig, and these IFNs possibly induce endometrial IFN-stimulated genes (ISG) for endometrial remodeling and modulation of the maternal immune system. Interestingly, the estrogen production of the conceptus is thought to induce interferon regulatory factor 2 (IRF2) in a cell-specific manner, namely in the LE only, while restricting the expression of IRF1 to the stroma as shown in sheep [20]. In mares, the mechanisms of MRP are not completely understood to date. The equine conceptus is enveloped by a tough glycoprotein capsule of spherical shape. The conceptus migrates continuously throughout the uterine lumen between days 6 and 16 after ovulation driven by uterine contractions. Then, the conceptus becomes immobilized ("fixed") at the base of one of the uterine horns. So far, the equine embryonic signal of pregnancy recognition is still unknown. It is known that the presence of a conceptus uncouples the oxytocin-induced release of luteolytic $PGF_{2\alpha}$ into the circulation [21]. None of the different secretory products released by the equine conceptus during early pregnancy has been shown to function as anti-luteolytic factor. The mechanosensation of the migrating conceptus is thought to play a role, since intrauterine application of water-filled plastic balls has been shown to prolong luteal life span in the mare [22]. Very recently, intrauterine administration of plant oils has been shown to prolong luteal life span, possibly because of fatty acids interfering with the PG metabolism [23].

Despite species-specific differences and peculiarities, in both bovine and equine females there is a high proportion of embryonic losses during early pregnancy as observed in humans [24–26]. Therefore, a comparative approach of studying endometrial function can help to characterize embryo-maternal communication strategies and determinants of endometrial receptivity, which might be, at least in part, conserved, and aid in understanding critical events [27,28]. Multiple factors account for pregnancy success, and very different strategies have evolved to achieve establishment and maintenance of pregnancy. It is thus a challenge to encompass receptivity at a molecular level both technically and also with regard to comparative data analysis.

2. Animal models for the study of early embryo-maternal communication

Suitable *in vivo* and *in vitro* approaches are necessary to disentangle participating factors important for successful recognition and establishment of pregnancy.

In general, working with the species of interest is most favorable due to species-specific differences mentioned above. Although

differences may be only small, a matter of quantity or timing, they could lead to wrong conclusions due to different use of inter- and intracellular pathways (i.e., for recognition of pregnancy, different hormone metabolites and cofactors in target cells, different ways of hormone metabolism kinetics and different types of placentation). For humans, adequate *in vivo* models are needed to disentangle fertility issues, as early pregnancy is very difficult to approach. But as there is no easily accessible species resembling all features of human reproductive physiology, specific characteristics predispose different animal models to suit best. The protein sequence homologies, sex steroids and similarities in embryo development and gene structure [29] suggest the use of cattle as a possible model for human reproduction [30]. In cattle, research with embryos is possible, which is limited in humans due to ethical and legal reasons. Bovine *in vitro* embryo production is routinely applied, and pregnancies are also efficiently obtained with embryos produced by embryo splitting and somatic cell nuclear transfer (SCNT). Indeed, identical twins generated by embryo splitting have been used as recipient cows for embryo transfer experiments allowing the characterization of embryo-induced transcriptome changes in the endometrium in the absence of the effects of different maternal genotypes [31].

Other reasons for choosing an animal model may be the number of offspring, but also the feasibility of applying genetic tools such as gene knock-in, knock-down, and knock-out, as well as the availability of genome information including reliable information on gene transcripts and other working tools (e.g., antibodies). For some of these reasons, rodents (mouse, rat), drosophila with respect to sperm storage [32], or amphibians (large and numerous eggs of *Xenopus*) may be extremely helpful models even if they are far from being physiologically comparable to humans or to farm animals.

For physiological questions it is detrimental to extrapolate singularized *in vitro* evidence to a complex organism in trying to identify causes and consequences of signals. But depending on the level of complexity, abstraction becomes necessary, because compensatory mechanisms and redundant pathways present *in vivo* may conceal an effect. Because these latter are features of organisms, a useful way towards understanding function is also to have *in vitro* abstraction, to understand principal pathways. For this, standardized cell lines provide a most homogenous background for recurring experimental settings, even if they do not completely resemble the physiological context from which they were originally taken. *In vitro* models that display many characteristics of a natural environment are mostly primary cell cultures. Disadvantages are a high, unknown day-to-day variation due to variable origin and the relatively short time period primary cells can be cultured. With these limitations, the development of suitable immortalized cell lines or culture of tissue explants displaying as much of physiological properties as possible is promising [33,34]. For the analysis of early pregnancy, *in vitro* models encompassing an *in vivo*-like environment are needed to study local paracrine effects (Fig. 1). Studies *in vivo* during the peri-conceptual period are challenging to approach due to difficulties in localizing an embryo of only a few hundred μm in diameter in a large size reproductive tract [35–37].

In vivo settings comprising a specifically atypical phenotype are also suitable as models for the identification of genes with important functions regarding fertility. Increased placentomes and fetal oversize are frequently detected following SCNT in cattle [38]. While initial pregnancy rates after transfer of SCNT embryos are similar to those after transfer of flushed embryos [39,40], a continued pregnancy loss occurs during the course of pregnancy. An incorrect reprogramming event of the nucleus, including epigenetic alterations, most probably causes SCNT pregnancy losses [41–43]. Interestingly, these aberrant phenotypes have been

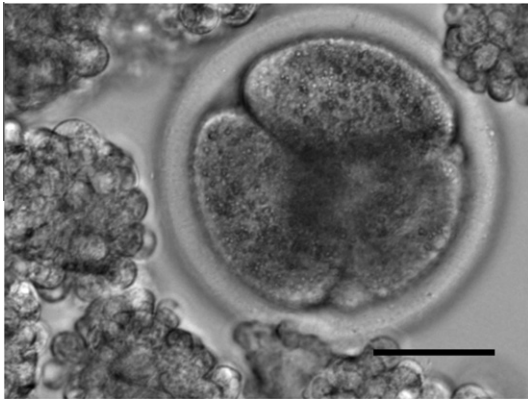


Fig. 1. Bovine embryo. A bovine embryo following *in vitro* fertilization is co-cultured *in vitro* with oviduct epithelia cells. By analyzing the transcriptome of the latter, early embryonic signals may be perceived. Bar length = 50 μm .

shown to be entailed by alterations in endometrial gene expression already apparent during the first three weeks of pregnancy [44,45]. Assuming an intensive and obligatory embryo-maternal exchange, inadequate signaling might in turn severely impact on embryonic and fetal development and may have negative effects on metabolic imprinting, eventually enhancing the susceptibility to chronic diseases in adult life [46].

Finally, the use of *in silico* modeling as possible predicting tool for biological processes becomes increasingly interesting to minimize the use of animals due to ethical reasons. The exponentially rising number of omics data available holds increasing information, which is only partly comprehended to date. A basic requirement is the availability of large data sets of as many different experiments as possible in a definite data format and suitable ways to access information from an infinite list of data sets.

3. Tissue sampling and material quality demands

In general, good biological models require a proper experimental design, adapted to the biological question to be resolved. In addition to technical variance, the biological alteration is the major source for variation in the results of gene expression studies and has to be considered for making an experimental design (i.e., biological replicates are required and the number of replicates depends on the variability) [47]. Biological variability can be caused for example by differences in tissue composition of collected samples, timing of sample collection and individual responses to treatments. In some studies pooling of samples has been applied to reduce costs and experimental effort. However, this leads to the loss of information on the biological diversification in the experimental system and can introduce bias into the results caused by biological outliers. On the other hand, pooling of tissue samples from the same experimental animal can be used to reduce variations in tissue composition in case of complex tissues [48]. In general, biological replicates should be analyzed to get significant information about gene expression changes in a biological system. Numbers of replicates strongly depend on the precision of the technological platform used for gene expression analysis and, for the most part, on the biological system. As many parameters as possible should be collected to characterize a biological system in order to explain the observed variation in the results obtained from transcriptome analyses.

There is a number of critical points that should be considered for the collection of defined tissue samples from animals. Particularly for the analysis of reproductive tissues, the collection from defined stages during the sexual cycle or during early pregnancy

is essential. This can be achieved, e.g., by synchronization of the cycle (injection of $\text{PGF}_{2\alpha}$ for induction of luteolysis) or ovulation and transfer of *in vitro* fertilized (IVF)-derived blastocysts at the respective day of the sexual cycle. Furthermore, determination of steroid hormone concentrations, evaluation of the status of the ovaries and developmental stage(s) of the conceptus(es) can be used as parameters for stage definition.

In addition to collection from defined stages, the performance of tissue sampling itself needs to be standardized to ensure i) short times for tissue or cell recovery and sampling (instability of RNA), ii) similar times for all biological replicates and iii) collection of representative tissue samples. In a recent study by Streyl et al. [48] tissue samples from bovine antepartum and intrapartum placentomes were collected by a systematic random sampling procedure to obtain representative samples from feto-maternal attachment zones. Furthermore, tissue composition was analyzed using quantitative stereology to estimate the percentage of epithelial cells of the individual samples [48]. Due to the relatively high variation in epithelial cell proportions and variation between different placentomes, a pooling strategy was applied using three samples from each of three placentomes per animal to reduce variations in tissue sample composition [48]. In a second study, biopsy samples from equine endometrium were analyzed by quantitative stereology to retrospectively control tissue sample composition since representative collection of biopsy samples is challenging [49]. Based on the results of quantitative stereology, samples with aberrant tissue composition can be excluded from the analysis, because large deviations in percentages of different cell types in a tissue sample is associated with deviations in gene expression patterns [49]. A more elaborate but most appropriate future approach is the separate sampling of cell types by laser microdissection as done in many different tissues including reproductive epithelia [50].

Finally, the integrity of the isolated RNA and also of the cRNA that is often used for hybridization to oligonucleotide microarrays has to be analyzed. Nowadays, quality of total RNA samples is preferentially characterized by automated microcapillary electrophoretic RNA separation, e.g., Agilent Bioanalyzer and Tape Station [51,52]. These techniques deliver RNA integrity values (RIN) [53] or ScreenTape Degradation Values (SDV) [52], respectively, as a quantitative value for the integrity of total RNA samples. This is much more reliable compared to quality assessment by standard agarose gel electrophoresis. Depending on the type of sample (cells from culture or tissues), RIN or SDV values can differ, mostly showing fully integer RNA for cell culture-derived samples and different degrees of partial degradation for samples derived from some types of tissues. The most important point for quality control of gene expression experiments is that samples should have RINs within a similar range, i.e., if a partial degradation (e.g., RIN 7–8) is present, it should be at least similar for all samples.

The quality of hybridization probes (cRNA) for oligonucleotide microarrays can also be analyzed using electropherograms from microcapillary separation by comparison of the size distribution of the cRNA produced by the linear amplification with T7 RNA polymerase. For Affymetrix 3' IVT arrays, integrity of biotinylated cRNA used for microarray hybridization can additionally be deduced from the probe level data with RNA degradation plots (Bio-Conductor package Affy). These plots show the average signal intensities for the most 5-prime to the most 3-prime probes for all probe sets. Since cDNA and cRNA synthesis are starting from the 3-prime end of the original mRNA, the number of labeled cRNA fragments decreases from 3-prime to 5-prime resulting in an increase of average signal intensities from 5-prime to 3-prime. The slope of the curves should be similar for all samples/arrays. Different slopes are due to deviations in cRNA fragment distribution and indicate differences in integrity of the RNA used for sample preparation.

4. Transcriptomics for analysis of differential gene expression

Mammalian genomes contain approximately 20,000–22,000 protein-coding genes and a growing number of genes for structural and regulatory RNAs. The number of individual transcripts encoded by mammalian genomes is significantly higher due to transcript isoforms arising from the same gene [54–57]. Furthermore, all the RNAs contained in a given tissue occur in very different abundances [54,58] making the parallel analysis of all RNAs contained in a sample a technical challenge. Transcriptome analyses are powerful tools for a system-wide analysis of cellular changes at the molecular level since most physiological processes are associated with complex changes in RNA concentrations. However, the ideal way would be to address the proteins themselves, as the functional players in the cell. But this is still limited by technical issues due to the considerably larger differences in abundances and the extremely diverse chemical properties of individual proteins making them only partially accessible for current proteome analysis techniques (mass spectrometry and 2D gel electrophoresis) [59]. In contrast, analytical approaches for the analysis of cellular RNAs have been developed for comprehensive profiling of mammalian transcriptomes [60–63]. Currently, the most powerful tools are based on hybridization (DNA microarrays) and on deep-sequencing (RNA-Sequencing, RNA-Seq), both able to generate comprehensive genome-wide expression profiles. The impact of these technologies can be seen in the exponential increase of PubMed abstracts containing the keyword ‘microarray’ (Fig. 2). With a delay of several years the microarray technology has been used in similarly increasing numbers of publications in domestic animal research (Fig. 2). The relatively new RNA-Seq technology provides some major advantages over the microarray technology, e.g., more precise information on absolute transcript levels, transcript variants, and currently not annotated transcribed regions and is used more and more in different biological applications [61,64]. Due to the nearly unlimited sensitivity, the RNA-Seq technology is particularly suited for the analysis of mammalian transcriptomes and enables the detection of rare transcripts in complex tissues, such as the endometrium. However, data from RNA-Seq experiments show also a number of biases related to sequence composition [65] and library preparation and problems similar to cross hybridization for microarrays in case of highly similar members of a gene family. A number of issues have to be considered for data analysis making RNA-Seq experiments much more complicated compared to microarrays. For domestic animals, the next-generation sequencing technologies will be extremely helpful for improving the current gene annotation, to define the entire transcriptome, and finally provide sequence information for the design of comprehensive genome-wide microarrays.

A comparison of the results derived from an RNA-Seq study and an Affymetrix microarray study of bovine endometrium at day 18 of pregnancy is shown in Table 1 (Bauersachs (2011), unpublished results). The same RNA samples were used for both studies. The comparison revealed a consistent overlap between the results but many more differentially expressed genes (DEGs) for the RNA-Seq data, which is consistent with results found in other studies [66].

In humans, a plethora of microarray studies of endometrial tissue samples has been published. The first transcriptome studies compared biopsy samples collected during the window of implantation time with samples from the proliferative phase or the early secretory phase [67–69]. These studies provided remarkable insights into endometrial maturation and preparation for implantation [70]. Based on data from a similar study, Díaz-Gimeno et al. developed an ‘endometrial receptivity array’ for prediction of endometrial receptivity [71]. In addition to studies of ‘normal’

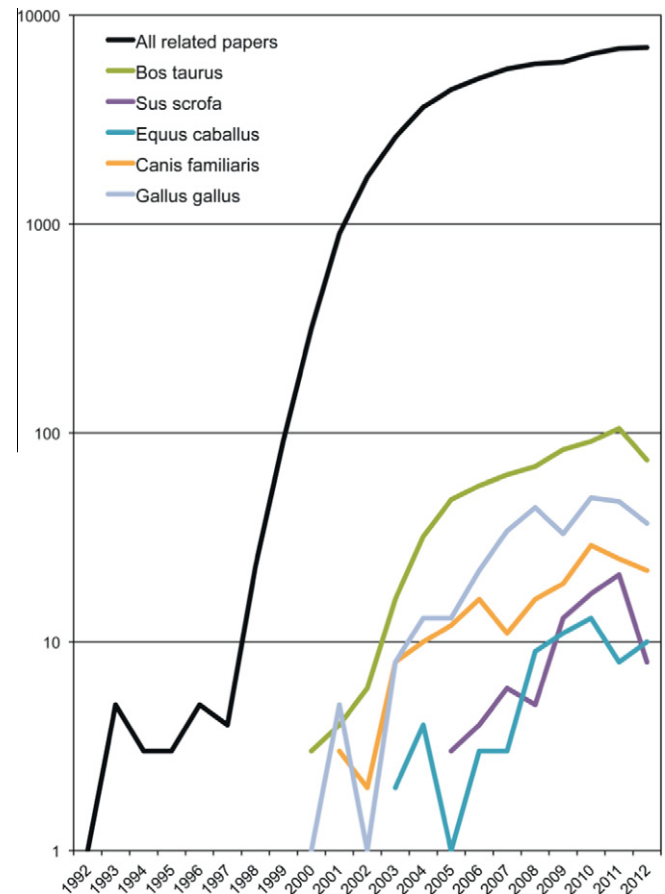


Fig. 2. Increase in citations. To generate comprehensive genome-wide expression profiles, hybridization (DNA microarrays) and deep-sequencing (RNA-Sequencing, RNA-Seq) are currently used. The numbers of PubMed abstracts containing the keyword ‘microarray’ or ‘microarray’ and a livestock species e.g., ‘Bos taurus’ have exponentially increased over the last decade. With a delay of several years, the microarray technology has been used in similarly increasing numbers of publications in domestic animal research. Vertical axis is in log scale.

cyclic endometrium, natural cycles and cycles during stimulated ovulation were compared [72–75]. Furthermore samples from women with unexplained infertility have been compared to normal fertile endometrium [76,77]. Although so many studies have been performed for similar stages of human endometrium, there is actually no true meta-analysis of these data. Comparison of the results (differentially expressed genes) of similar studies revealed rather poor overlaps. There are some attempts to collect endometrial gene expression data and to provide genes critical for endometrial receptivity (<http://www.endometrialdatabase.com/edb/>, [71]). However, the main problem is that different microarray designs, annotation versions, and strategies for data processing were used and there are no consistent standards for data presentation and deposition of complete data sets in public repositories. These issues make a meta-analysis nearly impossible.

Meanwhile, a number of microarray studies has been performed for domestic animals such as sheep, cattle, pig and horse, on different reproductive tissues [49,62,78–81] to characterize regulatory processes underlying establishment and maintenance of pregnancy. Particularly for cattle, various stages of the peri-implantation phase have been investigated by different groups, namely day 13 [82,83], day 15 [84], day 16 [82], day 17 [85], day 18 [31,84,86] and day 20 [87]. A comparison of the results of these studies showed a consistent overlap of the identified differentially expressed genes [84]. Most recently, a study based on an RNA-Seq

Table 1
Comparison of Illumina RNA-Seq and AffymetrixGeneChip data. A transcriptomic analysis of pregnant vs non-pregnant endometrium at day 18 of pregnancy was undertaken using identical samples for RNA-Seq and Affymetrix analyses. The data sets of the resulting differentially expressed genes were compared.

	Number of genes	%
RNA-Seq (false discovery rate 1%, Fold Change ≥ 2)	664	
Affymetrix (false discovery rate 1% Fold Change ≥ 2)	336	
Differentially expressed genes of RNA-Seq analysis not represented on Affymetrix array	218	32.8
Differentially expressed genes of RNA-Seq analysis not detectable with Affymetrix (of RNA-Seq DEGs)	37	5.6
Differentially expressed genes of Affymetrix analysis found as differentially expressed with RNA-Seq	278	93.6

analysis of the day 16 bovine conceptus and corresponding pregnant endometrium revealed a comprehensive list of genes expressed in the conceptus coding for potentially secreted molecules that interact with receptors on the endometrium and vice versa during the window of maternal recognition of pregnancy [88]. In addition, endometrial gene expression during early pregnancy has been compared between fertile and subfertile dairy cow strains [89], under the influence of negative energy balance in postpartum dairy cows [90] and between cloned and IVF embryos [44,45]. Altogether, the transcriptome studies of bovine endometrium during early pregnancy and during the estrous cycle [91,92] provide a rich resource for future studies focused on specific genes and pathways suggested as particularly important for endometrial receptivity and for recognition and establishment of pregnancy.

So far only endometrial tissue or biopsy samples, which contained all compartments of this complex tissue, namely LE, GE, stroma (fibroblasts and other cells, e.g., immune cells) and blood vessels (endothelial cells), have been analyzed in previous transcriptome studies. However, results of other groups and our own results showed differences in responses to hormonal control and signals of the embryo between different compartments. For example, a comparison of the results of two studies comparing similar stages of cyclic endometrium (day 7 and day 14) using the same Affymetrix bovine microarray [93,94] revealed only a very small overlap, not higher than expected by chance. This small overlap 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, endometrial samples collected after slaughter in Forde et al. [82] and cytobrush samples in Salilew-Wondim et al. [93]. The cytobrush technique mainly yields cells from the luminal surface, i.e., LE. Since the LE accounts only for a small proportion of endometrial tissue samples, specific changes may be hidden when analyzing entire endometrial samples. The cell type-specific differences are most likely associated with the down-regulation of PGR in LE and superficial glands during the luteal phase [95]. The same holds true for the comparison of tissues with differing cellular composition. These results indicate that one of the future tasks for gene expression studies will be the separate analysis of endometrial compartments by laser microdissection. Although the challenges of microdissection are to obtain sufficient amounts and good quality of RNA for unbiased hybridization experiments, this pre-analytical sampling refinement will lead to a better understanding of interaction between different compartments and their regulatory processes.

5. Downstream analysis of single candidate genes – endometrial functions

In general, differential gene expression allows the presumption of gene products involved in a particular function and the generation of hypotheses. But unfortunately, descriptive data are limited, because it is challenging to disentangle causes from concomitant incidences, which are independent. In the context of biological

communication, it is not at all obvious to distinguish signals as initial setters from those that are responders, or simply secondary effects without further function. The deduction of interesting target genes from a large number of possible candidates is thus a demanding task, as it bears the risk of missing important players.

Comprehensive descriptions of physiological processes in high resolution with respect to time and spatial distribution are necessary to generate proper conclusions. One example is the study of the uterine milk proteins, SERPINA14, basic glycoproteins belonging to the serpin superfamily of serine peptidase inhibitors. Initially described in the 1980s, they have been characterized as the major secretory proteins expressed in the endometrium during pregnancy in ruminants [96,97]. Although they were not the predominant proteins during pregnancy in the cow, the presence of substantial amounts of SERPINA14 in bovine uterine histotroph during pregnancy was demonstrated [98]. The secretion was shown to be P₄-dependent, and the immunosuppressive action of P₄ was mainly attributed to SERPINA14. In one of our first studies analyzing the differential gene expression of pregnant versus non-pregnant bovine endometrium, SERPINA14 mRNA appeared to be slightly up-regulated during pregnancy [31], however a much more pronounced difference was observed by comparing endometrium of the secretory with the luteal phase [91]. Strikingly, this showed the opposite of what we had expected, since an increase during estrus is pointing towards estrogen-dependent up-regulation. An in-depth analysis at the protein level further confirmed that SERPINA14 indeed appeared prominently at estrus due to apical expression in the deep glandular epithelium [99]. Other than a species-specific peculiarity between ovine and bovine, it turned out that most probably the timing of sampling with respect to the estrogen peak had led to the missed assessment in sheep, because SERPINA14 appeared and disappeared in only a very narrow window. Thus, next to its function during later pregnancy, the analysis contributed to setting SERPINA14 in context with (a) a possible marker for ovulation and (b) a possible candidate gene for interacting with approaching sperm.

Inconsistencies between transcription, translation and post-translational modification may limit the extrapolation of biological function from transcriptome data. Discrepancies can only partially be explained by secretion products, which do not appear in the same cell types analyzed on transcriptome and proteome level. Here, analyzing in parallel the secretome of e.g., glandular epithelial cells in the secretory lumen, the uterine histotroph, together with the transcriptome and proteome of these epithelial cells seems appropriate. Further, methodological challenges for downstream analyses include various analyses by means of localization within a tissue.

Furthermore, interesting factors, which drive biological events, may be metabolic intermediates, hormones, cytokines, which may not appear as differentially regulated on the transcriptional level, since enzymes involved in metabolism of nutrients and other non-protein signaling molecules as well as peptide hormones and cytokines themselves could be regulated at the protein level or just by secretion after cell activation. In this context, we undertook a metabolomic approach of the uterine fluid representing the embryo-maternal interface and investigated the precise nutrient

composition concerning 41 amino acids (AA) and AA derivatives during the time of extensive trophoblast growth by high sensitive liquid chromatography tandem mass spectrometry [100]. In addition, the transcript abundance of endometrial transport systems was as well analyzed. As the conceptus elongates tremendously, a substantial amount of nutrients must be provided by the transport from the maternal circulation into the uterine fluid rather from *de novo* synthesis of the conceptus or secretion into the uterine lumen. However, a differentiation whether non-essential AA and derivatives in the uterine fluid derive from either the mother or the developing conceptus is not possible. It is known that pre-implantation embryos release metabolic products into the uterine lumen [101].

Concordantly with results obtained from studies in the ewe [102], we observed an increase in most essential and non-essential AA in the uterine lumen of pregnant animals during pre-implantation [100]. Most excitingly, the increase in the pre-implantation phase was detected for the branched chain AA, most pronouncedly L-isoleucine. Essential AA are not only required for protein synthesis, but are also necessary for rapid growth and cell differentiation. Since the increase of most AA during the phase of embryo elongation was not observed in the uterine fluid of non-pregnant cattle, a direct or indirect induction of the AA transport into the uterine lumen by components provided by the conceptus may reasonably be presumed. Interestingly, the transcript abundance of AA transporters analyzed was not affected by pregnancy status, except for the lysosomal His and peptide transporter *SLC15A3* [100], a known IFNT dependent gene. Analyses of AA concentrations in the histotroph of SCNT pregnancies were carried out, since previous studies had shown alterations of the endometrium transcriptome profile at day 18, most probably due to aberrant signaling of cloned embryos [44,45]. Most astonishingly, the AA abundance in the histotroph of SCNT pregnancies was reduced as compared to IVF pregnancies, although the intra-luminal concentration of IFNT was the same [103]. Particularly, the concentration of essential AA was reduced, and these originate from the endometrium only. Thus, the conclusion that can be drawn is that (a) the AA abundance increased during early pregnancy in the uterine lumen without accompanying changes of transporter transcripts, and (b) embryonic signals other than IFNT induced either directly or indirectly maternal physiological processes that in turn increased the abundance of luminal AA during pregnancy in cattle.

6. Upstream analysis of signal fingerprints – endometrial sensing

Large-scale transcriptome data may not only describe physiological changes that lead to downstream protein translation and function. They can also mirror most valuable information regarding present events. This means that transcriptome expression differences can hold as fingerprints that allow the interpretation of stimuli that occur. The expression fingerprint is independent from further downstream reactions that take place. Rather, the vast number of changes, that do not necessarily need to be large, may be taken together to condense information about signaling responses.

Hierarchical clustering or principal component analyses (PCA) may be appropriate to condense information, as it has successfully been proposed for a number of applications including e.g., biomarkers of circulating tumor cells, tumor progression or prescreening of anabolic misuse [104,105]. A PCA can enhance the signal to noise ratio for entangling subtle gene expression changes by combining a large set of differentially expressed genes of low fold-change.

With respect to endometrial function, this approach can hold true for the analysis of yet unknown signaling molecules secreted

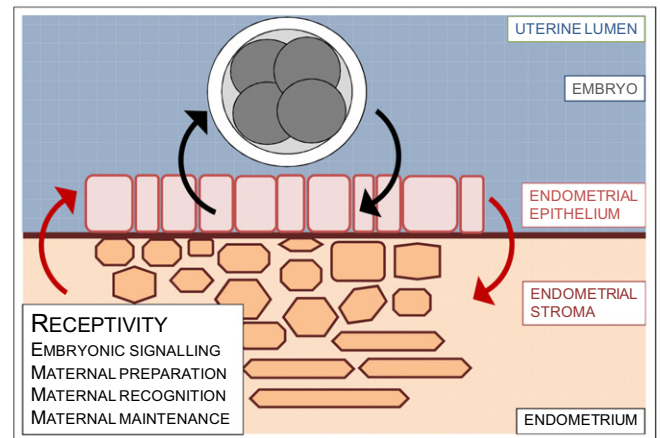


Fig. 3. Endometrial sensing. Uterine receptivity is a prerequisite for embryonic development. It comprises of a complex interplay of factors derived from the embryo acting on the endometrial epithelium, and maternal factors acting on the developing embryo, respectively (black arrows). In addition, endometrial epithelium and the underlying stromal cells exchange signals in a paracrine manner (red arrows). Aberrant embryonic signaling may be perceived as transcriptomic fingerprint of endometrial tissue following *in vitro* co-culture.

by the trophoblast. For example, the molecules that equine embryos are secreting may be deduced from its endometrial fingerprint. Functional groups connected to estrogen signaling, prostaglandin metabolism, and vascular remodeling appeared as overrepresented pathways in pregnant endometrial transcriptome analyses, pointing towards the plurality of signals that are secreted [49]. If singular factors, e.g., estradiol-17b, PGE₂ or others would be applied to uteri of mares, further transcriptome data could be subtracted from the physiological datasets and differences in expression profiles would substantiate the embryo-specific signaling.

Cloned embryos have been shown to induce differential gene expression in recipient mothers prior to implantation different from the changes induced by *in vitro* produced and *in vivo* embryos [44,45]. Since pregnancies with cloned embryos can lead to birth of a healthy calf, the endometrium may have the ability to adapt to inconsistencies of embryonic signaling within a certain range. This would be useful for non-invasive monitoring of embryo quality. Only recently, cultured endometrial cells from women with recurrent miscarriage showed a peculiar response towards high- and low-quality human embryos compared to those from fertile women [26]. The cultured stromal cells of patients with recurrent miscarriage did not distinguish between the quality of the embryo, and their migratory activity was abnormally high in case of the presence of a low-quality embryo. This non-discriminative migration was considered as biomarker for identifying 'selection failure'. Following this approach, a molecular setup could implement an *in vitro* cell culture system of reproductive epithelium and co-cultivate embryos prior to transfer. The read-out of transcriptional changes could infer whether an adequate signaling had taken place or not (Fig. 3). According to the quiet embryo hypothesis in favor of metabolically less active embryos [106], large transcriptional changes would indicate perturbations further to be specified.

7. Conclusion

In mammals, species-specific differences in mechanisms of embryo recognition and establishment of pregnancy hold major challenges in molecular recapitulation. However, by unraveling the difficulties, comparative analyses of endometrial gene expression offer great opportunities to find homologous changes and distinct differences to draw conclusions for further species including

humans, where analyses are difficult to perform. Analysis of the endometrial transcriptome may not only increase the knowledge of potentially important molecules involved in establishment of pregnancy, but is also very useful for the assessment of effects of environmental factors influencing reproductive performance. Specific attention has to be drawn to advantages and limitations of different experimental models, the process of sampling, the inconsistencies between gene transcript and protein, as well as data evaluation due to inherent limitations.

Holistic data sets offer great potential to identify molecular pathways overlooked in hypothesis-driven candidate approaches. They provide comprehensive information for the generation of new hypotheses. The greatest advantage is probably the information currently hidden due to the reduction of data complexity. New bioinformatics tools comparing the increasing number of large data sets are promising to extract new inherent biological information. Finally, transcriptome studies offer the possibility to deduce molecular fingerprints of elapsed events, which may either unravel the causal signals or allow the use as molecular markers.

Acknowledgements

Our studies were supported by the Deutsche Forschungsgemeinschaft (FOR478, FOR1029), the German Ministry for Education and Research (BMBF; programs FUGATO and FUGATO-plus; projects FERTILINK and COMPENDIUM) and in part by the Society for Reproduction and Fertility.

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