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The alterations in endometrial and myometrial transcriptome at the time of maternal recognition of pregnancy in pigs

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ABSTRACT

The maternal recognition of pregnancy in pigs occurs around day 12–13 of gestation. During this period embryo-derived signals act on the endometrium and the myometrium to trigger tissue-specific responses that lead to proper implantation and maintenance of pregnancy. The present study utilized gene expression microarrays to examine embryo-induced alterations in porcine endometrial and myometrial transcriptome on days 12–13 of pregnancy. It has been confirmed that the endometrium and the myometrium, in response to embryonic signals, express unique set of genes. However, a key discovery of this study is the fact that genes that encode crucial factors involved in maternal recognition of pregnancy and preparation of uterus for implantation are regulated similarly in both tissues. Genes responsible for PGs synthesis (*PTGES2*, *PTGR2*), uterine development and remodeling (*HOXA10*, *GRB10*, *MTOR*) and innate immune response and immunomodulation (*NFKBIA*, *TRAFD1*) were regulated similarly in the endometrium and the myometrium. This suggests that in pigs these tissues act in sync to support embryos during peri-implantation.

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1. Introduction

It is known that the process of maternal recognition of pregnancy in pigs takes place around day 12 of pregnancy (Bazer et al., 1994; Geisert et al., 1982a). According to the classical theory of maternal recognition of pregnancy in pigs blastocysts manifest their presence in the uterus of the mother by secretion of estrogens (Bazer et al., 1994; Geisert et al., 1982a). Nowadays, it is also confirmed that apart from estrogens, porcine conceptuses secrete cytokines — namely interferons (Cencič and LaBonnardière, 2002) and interleukin 1β (Ross et al., 2003). Altogether, embryo-derived factors constitute embryo-maternal cross-talk by acting as signals for maternal recognition of pregnancy and modulators of uterine secretory activity. The interaction of embryo with the endometrium is the first prerequisite for triggering the process of implantation which determines the success of pregnancy (Geisert et al., 1982b). In order to maintain the pregnancy, endometrial and myometrial secretory activity needs to be modulated in a way that facilitates protection of corpus luteum (CL) from luteolysis (Christenson et al., 1994; Franczak et al., 2006). In pigs prostaglandins (PGs) are uterine originated factors that determine the life-span of CL (Akinlosotu et al., 1986; Moeljono et al., 1976).

Later during the course of pregnancy, the role of uterus is to provide an optimal microenvironment for embryo development. This requires a multilateral secretory activity – apart from aforementioned prostaglandins, endometrium is also a source of steroid hormones, enzymes, transport proteins, neuropeptides, growth factors, chemokines and cytokines (Bazer et al., 2012; Okrasa et al., 2014). The extensive work by Franczak and colleagues provided evidences that also porcine myometrium is a source of steroid hormones and prostaglandins (Franczak, 2008; Franczak and Kotwica, 2008; Franczak et al., 2014a, 2014b) and therefore this tissue actively supports the maintenance of pregnancy in pigs.

Considering the potential major role of myometrium in creation of proper microenvironment for developing embryo, the full understanding of the process of maternal recognition and maintenance of pregnancy cannot be achieved without analyzing endometrium and myometrium action in conjunction.

Abbreviations: ACTB, β-actin, gene; *ANP32B*, Acidic leucine-rich nuclear phosphoprotein 32 family member B, gene; BLAST, Basic Local Alignment Search Tool; *CCR1*, Chemokine (C-C Motif) Receptor 1, gene; CEM, Conceptus Exposed Medium; CL, Corpus Luteum; Cy3, Cyanine 3; Cy5, Cyanine 5; *GAPDH*, Glyceraldehyde 3-Phosphate Dehydrogenase, gene; GO CC, Gene Ontology Cellular Component; *GRB10*, Growth Factor Receptor-Bound Protein 10, gene; *HOXA10*, Homeobox A10 protein, gene; *IGF2*, Insulin-like Growth Factor 2, gene; IL1β, Interleukin-1β; LH, Luteinizing Hormone; LHCGR, Luteinizing Hormone/Choriogonadotropin Receptor; *MTOR*, Mechanistic Target Of Rapamycin, gene; *NFKBIA*, Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells Inhibitor, Alpha, gene; *NOS*, Nitric Oxide Synthase, gene; *ODC1*, Ornithine Decarboxylase, gene; PGE2, Prostaglandin E2; PGF2α, Prostaglandin F2α; PGs, Prostaglandins; *PTGES2*, Prostaglandin E Synthase 2, gene; *PTGR2*, Prostaglandin Reductase 2, gene; PTGS2, Prostaglandin Synthase-2; *SMAD4*, SMAD Family Member, gene; TLR, Toll-like Receptor; TNFα, Tumor Necrosis Factor α; TRAFD1, TRAF-type zinc finger domain containing 1; *ZNF280D*, Zinc Finger Protein 280D, gene; *ZNF445*, Zinc Finger Protein 445, gene.

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In this paper, we present the results of joint analysis of porcine endometrial and myometrial transcriptome during the time of maternal recognition of pregnancy, i.e. during days 12 to 13 of pregnancy. Pregnancy-specific gene expression profiles were obtained by comparison of profiles in tissues harvested from pregnant with non-pregnant gilts using the Porcine (V2) Gene Expression Microarrays 4 × 44 (Agilent Technologies, USA). The aim of the study was to evaluate if the endometrium and the myometrium response to embryo-derived signals similarly or differentially. The specific objectives of the study were: 1) analysis of alterations in porcine endometrial transcriptome during maternal recognition of pregnancy, 2) analysis of alterations in porcine myometrial transcriptome during maternal recognition of pregnancy and 3) comparison of the endometrial and myometrial gene expression profiles.

2. Material and methods

2.1. Ethics statement

All experiments were approved by the Animal Ethics Committee, University of Warmia and Mazury, Olsztyn, Poland.

2.2. Animals and tissues collection

Post-pubertal gilts (Large White × Polish Landrace, 90–110 kg) slaughtered on days 12 to 13 of pregnancy ($n = 4$) or the estrous cycle (n = 4) were used in the experiment. The estrus behavior of the gilts was observed in the presence of an intact boar throughout two consecutive cycles. The onset of the second estrus was designated as a day 0 of the estrous cycle. Gilts assigned to the pregnancy group were naturally bred on the second day of estrus. Pregnancy was confirmed by recovery of embryos from uterine horns by flushing with sterile saline (20 ml). The stage of the estrous cycle was confirmed by monitoring the morphological changes of the ovaries and CLs (Akins and Morrissette, 1968). Instantly after slaughter, uteri were excised and sections of the middle part of uterine horns were opened longitudinally on the mesometrial surface. The endometrium and the perimetrium were separated from the myometrium by careful scraping using a scalpel blade. Small fragments of the endometrium and the myometrium were then minced, snap frozen in liquid nitrogen and stored at −80 °C. Precision of separation of the endometrium and the myometrium was verified under a dissecting microscope and histologically.

2.3. RNA isolation and microarray data analysis

RNA isolation, evaluation and the DNA microarray study were performed as described in detail in the previous paper (Franczak et al., 2014a, 2014b). Briefly, RNA was extracted using a Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA) along with DNAse (RNase free DNAse Kit, Qiagen, USA) treatment to digest any remaining DNA. RNA integrity was evaluated using a 2100 Bioanalyzer (Agilent Technologies, USA). RNA integrity number (RIN) was calculated for each sample using Agilent 2100 Expert software. Samples with a RIN value of 7.5 and more were further processed. The Porcine (V2) Gene Expression Microarrays 4 × 44 (Agilent Technologies, USA) were utilized in this study. The arrays were processed according to the Two-Colour Microarray Based Gene Expression Analysis protocol v. 6.7.

Total RNA was amplified and labelled with fluorochromes using classical dye-swap approach. Labelling was performed with use of Low Input Quick Amp Kit (Two-Color) (Agilent Technologies, USA). After purification of the labelled RNA (Qiagen RNeasy Kit), RNA yield (nanograms of complementary RNA (cRNA) and specific activity (picomoles of Cy3 or Cy5 per microgram of cRNA) were quantified using an Infinite 200 PRO plate reader equipped with a NanoQuant plate (Tecan Group, Germany). Labelled cRNA was then fragmented, mixed with hybridization buffer, and placed on the microarray slide. Two differentially labelled cRNA samples (obtained from pregnant and cyclic animals) were placed on each array ($n = 4$) in a balanced block design. The same procedure was used for the endometrium and the myometrium. The use of four independent biological replicates per group, in case of both tissues, allowed to obtain an experiment power of 80%, and a false discovery rate of 0.1%, according to the sample size calculation method described by Hu et al. (2005). The microarrays were then incubated for 17 h at 65 °C in an Agilent hybridization oven, dissociated from the hybridization chamber and washed twice in GE wash buffer (Agilent). After the wash step, the slides were scanned using Agilent's High Resolution C Microarray Scanner at the settings recommended for the 4 × 44 K array format. The images obtained after scanning were analyzed using Agilent Feature Extraction software v. 10.5.1.1. Analysis included filtering of outlier spots, background subtraction from features and dye normalization (linear and LOWESS).

2.4. Differentially expressed genes

The data obtained after extraction was further analyzed using GeneSpring GX 11.0.2 (Agilent, USA). The aim of the analysis was to determine which genes were differentially expressed in the endometrium and the myometrium isolated from pregnant and cyclic pigs. The list of differentially expressed genes was obtained after moderated *t*-test statistical analysis with the *p*-value set to 0.05. Subsequently, this list was subjected to fold-change analysis with cut-off for upregulation or downregulation set to 1.2-fold. This approach was based on previous experience (Franczak et al., 2014a, 2014b). In cases when a statistically altered gene was represented on the Agilent's Porcine V2 microarray by multiple probes, the mean fold-change was calculated and reported. The full data set is presented in Supplementary Table 1. Probe sets for which both upregulation and downregulation was detected were excluded from further analyses. The list of differentially expressed genes was then manually enriched via alignment of the unknown gene probe sequences with the porcine transcriptome using Basic Local Alignment Search Tool (BLAST). Significant differences in gene expression were determined via Student's *t*-test. The differences were considered statically significant at $p \leq 0.05$.

2.5. Enriched gene ontology terms analysis

Lists of differentially expressed genes were uploaded to The DAVID 6.7 - Database for Annotation, Visualization and Integrated Discovery Classification System (da Huang et al., 2009a, 2009b) to analyze their functional affiliations. Ontologies were assigned independently to upregulated and downregulated gene lists obtained for each examined tissue. Annotations were limited to *Sus scrofa* and genome of this species was used as a background.

2.6. Comparison of the endometrial and myometrial gene expression profiles

The obtained lists of upregulated and downregulated genes in the endometrium from days 12 to 13 of pregnancy were compared with the list of upregulated and downregulated genes in the myometrium from the same days of pregnancy, respectively. Ambiguous genes (both upregulation and downregulation reported) were excluded from analyses. For the purpose of comparisons, Venn diagrams for upregulated and downregulated genes in the endometrium and the myometrium were constructed with use of the Venny online tool (Oliveros, 2007).

2.7. Quantitative real-time PCR analysis of gene expression in the endometrium and the myometrium

Thirteen genes with the same pattern of expression in the endometrium and the myometrium, six upregulated (*CCR1*, *GRB10*, *HOXA10*, *NFKBIA*, *PTGES2*, *ZNF280D*) and six downregulated (*ANP32B*, *LHCGR*,

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