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In vitro effect of orexin A on the transcriptomic profile of the endometrium during early pregnancy in pigs

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ABSTRACT

Orexin A belongs to the group of hypothalamic-derived peptides that are involved in a number of processes, such as regulation of energy metabolism, control of food intake and regulation of the reproductive system, by influencing the hypothalamic-pituitary-ovarian axis. Orexin A is also present in the endometrium, myometrium and placenta, which indicates that it may function as an important local regulator of the reproductive functions. The aim of this study was to explore the effects of orexin A on global gene expression in the endometrium of pigs during early gestation, on days 15 to 16 of pregnancy (implantation period). Orexin A altered the expression of 1,242 genes. In this group, 1,104 genes had a fold change greater than 1.2 ($P < 0.05$). In the group of genes with a fold change that was greater than 1.2, the expression of 457 genes were increased and 647 decreased because of the effects of orexin A. An analysis of the interactions between differentially expressed genes demonstrated that orexin A interacts with genes that potentially encode intracellular signalling pathways and factors regulating reproductive functions in the endometrium. The data from the present study indicate that orexin A affects a number of genes and processes in the endometrium of pregnant pigs and may be regarded as an important regulator of implantation, depending on maternal nutritional status.

1. Introduction

The correlation between the reproductive success of animals and their metabolic and nutritional status has been well documented. Orexins are hormonal factors that probably exert the greatest effects on reproductive functions in response to the animals' metabolic status.

The factors of the orexin system (orexin A, orexin B and the respective receptors) have been identified in the reproductive structures of many animal species. There is a presence of orexins and the respective receptors in rat and pig ovaries and in pig uteri, conceptuses and trophoblasts (Silveyra et al., 2007; Nitkiewicz et al., 2010, 2012; Nitkiewicz et al., 2014; Smolinska et al., 2014). The orexin system is also functional in human, cat and dog placentae (Nakabayashi et al., 2003; Dall'Aglio et al., 2012). The results of

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previous studies revealed the relationship between orexins and orexin receptors concentrations and the phase of the oestrous cycle or early pregnancy, which indicates that functions of the orexin system in the uterus depend on the local hormonal status (Nitkiewicz et al., 2012; Smolinska et al., 2015).

There was a modulatory effect of orexin A (OXA) and orexin B (OXB) on the pig endometrium and myometrium in previous studies (Kiezun et al., 2017; Kaminski et al., 2018). Findings indicate both OXA and OXB modulate the expression of genes encoding for important steroidogenic enzymes – cytochrome P450c17 (CYP17A1) and cytochrome P450 aromatase (CYP19A3), as well as the secretion of steroid hormones such as oestradiol, oestrone and testosterone by pig endometrial and myometrial tissues during early gestation and on days 10 to 11 of the oestrous cycle. Considering these previous results, we hypothesised that orexins may alter the pig endometrial transcriptomic profile during the early gestation period. To test this hypothesis, the effects were assessed of OXA on global gene expression in the pig endometrium during implantation (days 15 to 16 of gestation), which is probably the phase of early gestation when there are the most changes in endocrine and physiological tissue functions.

2. Materials and methods

2.1. Animals and tissue collection

Four mature gilts (Large White x Polish Landrace) were used in the study ($n = 4$). All individuals were from private breeding farm (L. Wisniewski breeding farm, Krolikowo, Poland). The gilts were at the age of 7 to 8 months and the weight of 120 to 130 kg. All animals used in the study were pregnant and at the 15 to 16 day-stage of gestation. The day after coitus was considered to be the first day of gestation. Insemination was performed on days 1 to 2 of the oestrous cycle. Pregnancy was confirmed by the presence of conceptuses. At 15 to 16 days after coitus, gilts were sacrificed. Pigs were slaughtered by qualified abattoir personnel. Uteri collected after slaughter were placed in ice-cold PBS supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin and transported to the laboratory on ice within 1 h for conducting *in vitro* tissue culture. All studies were conducted in accordance with ethical standards of the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn (ethical approval: 91/2011/DTN).

2.2. Tissue cultures

Endometrial explant procedures were performed by modifying the technique described by Franczak (2008) using the modifications described by Smolinska et al., 2016a. The endometrial explant tissues obtained from the middle of uterine horns were cut into small, irregular slices (3 mm of thickness, 100 mg ± 10%). Tissue explants were washed three times in medium M199 (Sigma-Aldrich, USA). The tissue explants were placed in the separate sterile culture vials with 2 mL medium 199 containing 0.1% BSA (MP Biomedicals, USA), 5% dextran/charcoal-stripped new-born calf serum (Sigma-Aldrich), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Cultures were pre-incubated for 2 h (37 °C, 95% O_2, 5% CO_2). To determine the influence of OXA on the global gene expression in the endometrium, slices were treated with OXA (Sigma-Aldrich) at the concentration of 10 nM and incubated for another 24 h at the same conditions. Concentration of the hormone was chosen based on findings in previous studies Barreiro et al. (2004); Sasson et al. (2006) and Small et al. (2003). Control tissues were incubated without any treatments being applied. All cultures were prepared in duplicates in four separate experiments ($n = 4$). Only one, random explant from each duplicate (treatment or control group) was used in further analysis. The endometrial tissue explants were frozen and stored at −80 °C for further analyses. The viability of slices was monitored by quantifying lactate dehydrogenase (LDH) activity in media after 2 h of pre-incubation as well as at the end of the treatment period. The release of LDH was performed using a Liquick Cor-LDH kit (Cormay, Poland) following the manufacturer's instructions. The activity was compared of LDH during the culturing of the endometrial slices to its activity in medium obtained after breakage of endometrial cell walls by homogenisation (positive control for causing cell death and the maximal release of LDH). Mean LDH activity in cultured slices after the treatment period was 55.1 ± 4.5 U/L (1.8% of maximal release of LDH after cell homogenisation).

2.3. Total RNA isolation and quality control

Total RNA was extracted from tissues using peqGOLD TriFast isolation system (Peqlab, Germany) according to the manufacturer's instructions. The RNA purity and quantity were measured spectrophotometrically (Infinite M200 Pro, Tecan, Switzerland). The RNA quality was checked using RNA 6000 Nano Assay Kit (Agilent Technologies, USA) on an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA integrity number (RIN) score between 8 and 10 were used for microarray analysis and quantitative real-time PCR (qPCR) experiments. The RNA was stored at −80 °C until the microarray analysis.

2.4. Microarray analysis

Low Input Quick Amp, Two-Color kit (Agilent Technologies, USA) was used to amplify and label the total RNA. The RNA isolated from the control and OXA treated tissues was labelled with Cyanine-3 (Cy-3) and Cyanine-5 (Cy-5), respectively. Quantity and purity of cRNA were measured with NanoQuant plates on the Infinite M200 Pro plate reader (Tecan Group, Switzerland). There was mixing of 825 ng of Cy-3 and 825 ng of Cy-5-labeled cRNA (for each microarray). According to microarray manufacturer's protocol, cRNA was fragmented and mixed with hybridisation buffer. Subsequently, the mixture was added to Porcine (V2) Gene Expression Microarray 4 × 44 (Agilent Technologies, USA). Two differentially labelled samples (from OXA treated and non-treated, control

samples) were placed on each array (one slide, $n = 4$) in a balanced block design with dye swaps. To discount the dye bias effect observed in the dual-colour experiments, the study design included the alternate use of both dyes (dye swap), namely, in two microarrays the control probes were dyed by Cy-3 and the OXA-treated samples with Cy-5, whereas in another two microarrays, the control probes were dyed by Cy-5 and the OXA-treated, by Cy-3. Hybridisation was conducted in an Agilent hybridisation oven for 17 h at 60 °C. After the hybridisation process, the microarrays were washed and scanned at 5 μm resolution with an Agilent's High-Resolution C Microarray Scanner. The TIFF image, generated after the scanning process, was used for: data extraction and detailed analysis, background subtraction from features, and dye normalisations (linear and LOWESS); in the Feature Extraction Software (Agilent Technologies, USA).

2.5. Bioinformatic analysis

Gene expression data were analysed in GeneSpring GX 12 software (Agilent Technologies, USA) to identify the genes that were differentially expressed in pigs that were and were not treated with OXA. Gene transcripts for which there was an increased or decreased abundance were identified by fold change (FC) cut-offs greater than 1.2 and $P < 0.05$. The 1.2 FC cut-off was based on previous findings (Szeszko et al., 2016; Li et al., 2017; Zglejc et al., 2018). Normalised fluorescence intensity in animals treated and not treated with OXA was compared by Student's t-test. Fold change calculations were based on the mean abundance of gene transcripts in four biological replicates in pigs treated and not treated with OXA. Fold change values were calculated for replicates with differences in gene transcript abundance.

2.6. Gene ontology analysis

To explore functional class scoring in the resulting gene list, by means of gene ontology (GO) term enrichment analysis (Huang et al., 2009), a list of genes (DE-genes) for which there differential gene transcript abundance was uploaded to the Database for Annotation, Visualisation, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov). A modified version of Fisher's exact test was used to determine the level of significance.

2.7. Biological pathways analysis

The biological pathways were explored with the use of the DAVID database. The pathways were enriched with information from every individual gene, and total over-representation for each pathway represented in the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg) was computed. A modified version of Fisher's exact test was used to determine the level of significance.

2.8. Interaction network of genes with differential abundances of transcripts

From the DE-genes list with FC > 1.2, 27 genes were selected to create an interaction network: prolactin receptor (*PRLR*), luteinising hormone beta polypeptide (*LHB*), interleukin 1 beta (*IL1B*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1 (*HSD3B1*), GM2 ganglioside activator (*GM2 A*), cytochrome P450 family 21 subfamily A member 2 (*CYP21 A2*), transforming growth factor alpha (*TGFA*), C-X-C motif chemokine ligand 8 (*CXCL8*), prostaglandin-endoperoxide synthase 1 (*PTGS1*), colony stimulating factor 3 receptor (*CSF3R*), hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 2 (*HSD3B2*), insulin receptor (*INSR*), C-X-C motif chemokine ligand 1 (*CXCL1*), C-X-C motif chemokine ligand 3 (*CXCL3*), hydroxysteroid 17-beta dehydrogenase 8 (*HSD17B8*), corticotrophin releasing hormone binding protein (*CRHBP*), aminolevulinate dehydratase (*ALAD*), inhibin beta A subunit (*INHBA)*, fibroblast growth factor 7 (*FGF7*), cysteine and glycine rich protein 2 (*CSRP2*), cyclin dependent kinase inhibitor 1C (*CDKN1C*), nitric oxide synthase 3 (*NOS3*), gonadotrophin releasing hormone 2 (*GNRH2*), eukaryotic translation initiation factor 4B (*EIF4B*), glycoprotein hormones, alpha polypeptide (*CGA*), parathyroid hormone 1 receptor (*PTH1R*) and alkaline phosphatase, placental (*ALPP*). The selected genes are known to be associated with many processes involved in metabolism and reproduction. GeneMania Prediction Server was used to create the gene interaction network (Warde-Farley et al. (2012)). Networks were created on the base of known interactions: co-expression, co-localisation, genetic interactions, shared protein domains and physical interactions.

2.9. Microarray results validation

To confirm the microarray analysis results, the abundances of transcripts for 11 genes were analysed using quantitative real-time PCR method. One microgram of RNA was used in the reaction of reverse transcription in a total volume of 20 μl with 0.5 μg oligo(dT) 15 (Roche, Switzerland). The reaction was prepared using Omniscript RT Kit (Qiagen,USA) at 37 °C for 1 h and was terminated by the incubation at 93 °C for 5 min. Quantitative real-time PCR analysis was conducted using PCR System 7300 and Power SYBR Green Master Mix (Applied Biosystems, USA), as described previously (Szeszko et al., 2016). The PCR reaction mixtures contained 20 ng of cDNA, one of 13 primer pairs (forward and reverse): acetyl-CoA carboxylase alpha (*ACACA*) – 500 nM, activin A receptor type 2 A (*ACVR2 A*) - 500 nM, cytochrome P450 aromatase (*CYP19A3*) – 500 nM, cytochrome P450 family 51 subfamily A member 1 (*CYP51 A1*) – 500 nM, oestrogen receptor 1 (*ESR1*) – 100 nM, leptin (*LEP*) – 300 nM, lipin 1 (*LPIN1*) – 600 nM, peroxisome proliferator activated receptor alpha (*PPARA*) – 800 nM, prostaglandin F receptor (*PTGFR*) – 200 nM, prostaglandin reductase 2 (*PTGR2*)

– 500 nM, stearoyl-CoA desaturase 5 (*SCD5*) – 500 nM, β-actin (*ACTB*) – 500 nM, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) – 500 nM; 12.5 μl Power SYBR Green PCR Master Mix (Applied Biosystems), and RNase free water at the final volume of 25 μl. Real-time PCR reaction conditions, as well as forward and reverse primers sequences has been presented in Supplementary file 1. Negative controls were performed in which cDNA was substituted by water, or reverse transcription was not performed before PCR. All samples were prepared in duplicates. The specificity of amplification was tested at the end of the reaction by the analysis of melting-curve. Calculation of the relative abundances of transcripts for validated genes were conducted based on the comparative cycle threshold method (ΔΔCT), and normalised using the geometrical means of reference gene transcript abundance: *ACTB* and *GAPDH*. There were no significant differences in Ct values for reference genes between the treated and non-treated groups. To determine the differences in abundances of gene transcripts between OXA-treated and non-treated samples, a one-way ANOVA was used followed by use of the Fisher's LSD *post hoc* test. Statistical analyses were performed using the Statistica software (StatSoft Inc., Tulsa, OK, USA). Values for $P < 0.05$ were considered statistically significant.

3. Results

3.1. Genes with differential transcript abundance

A complete list of genes with differential transcript abundances between control groups and OXA-treated groups has been attached in Supplementary file 2. The table contains a probe set name, gene ID, fold change, the *P* value, the direction of change (up or down, when compared to the control) and the gene description with its accession number. Among 1,242 differentially expressed genes 1,104 of these had a fold change of greater than 1.2 ($P < 0.05$). Among the DE-genes with a fold change greater than 1.2, the transcript relative abundances of 457 genes was increased and for 647 genes was decreased as a result of treatment with OXA, when compared to the gene transcript relative abundances of the control group.

3.2. Gene ontology

The functional categorisation of the DE-genes were prepared using DAVID bioinformatic tools. Supplementary file 3 included the results of the entire DE-gene list (both, with increased and decreased gene transcript abundances; modified Fisher exact test $P < 0.1$). In Supplementary file 4, there is a list of the biological processes associated with DE-genes with both, increased and decreased transcript abundance.

Taking into consideration findings with the data analysis, there were 58 of the most important processes selected for the endometrial tissue. Among the selected processes, nine were enhanced and 51 were decreased (Table 1.). Analysis of list of DE-genes with the greater abundance of transcripts indicated there was a group of 16 DE-genes associated with *response to organic substance (GO:*0010033*)*. Another group of 15 DE-genes is associated with *cellular catabolic processes (GO:*0044248*)*. There was also a group of five DE-genes from this cluster that is involved in *cellular nitrogen compound catabolic processes (GO:*0044270*)*. Other biological processes that were affected by OXA treatment are *cellular response to stress (GO:*0033554*)*, with a group of 15 DE-genes with an increased relative abundance of transcripts. In the pig endometrium, OXA treatment also enhanced *alpha-amino acid metabolic processes (GO:*1901605*)* with the cluster of four genes. Another group of five DE-gene products is associated with *positive regulation of cytokine production (GO:*0001819*)*. In this cluster three DE-gene products are involved in *positive regulation of interferon-gamma production (GO:*0032729*)*.

Using the information from the ontology analysis for genes with decreased transcript abundance, the largest group of 55 DE-genes were associated with the *regulation of gene expression (GO:*0010468*)*. Among them 35 gene products that were associated with *positive regulation of gene expression (GO:*0010628*),* and 28 with *negative regulation of gene expression (GO:*0010629*)*. The next cluster of 51 DE-gene products influenced by OXA treatment was associated with *intracellular signal transduction (GO:*0035556*)*. In this group, 40 DE-genes was associated with *cell surface receptor signalling pathway (GO:*0007166*)*. From this group of 40 DE-genes, 19 were associated with the *enzyme linked receptor protein signalling pathway (GO:*0007167*)*, and nine were associated with the cell surface receptor signalling pathway involved in *cell-cell signalling (GO:*1905114*)*. Another group of gene products from the intracellular signal transduction cluster were involved in the *mitogen-activated protein kinase (MAPK) cascade* (*GO:*0000165; 15 gene products), *second-messenger-mediated signalling* (*GO:*0019932; 11 gene products), *inhibitor of kappa B kinase/ nuclear factor kappa-light-chain-enhancer of activated B cells (I-kappaB/ NF-kappaB) signalling* (*GO:*0007249; nine gene products), *protein kinase B signalling* (*GO:*0043491; seven gene products) and *NF-κB inducing kinase (NIK)/NF-kappaB signalling (NIK/ NF-kappaB)* (*GO:*0038061; three gene products). Another group of 49 inhibited DE-genes is responsible for *response to external stimulus (GO:*0009605*)*. In this cluster, there was a group of seven genes responsible for *response to nutrient levels (GO:*0031667*)*. The OXA treatment also inhibited a large group of 47 DE-gene associated with *regulation of signal transduction (GO:*0009966*)*. In this cluster, there were groups of DE-genes responsible for *regulation of intracellular signal transduction* (*GO:*1902531; 36 genes) and for *regulation of phosphatidylinositol 3-kinase signalling (PI3K)* (*GO:*0014066; seven gene products). Other biological processes modified by treatment with OXA are associated with *phosphorus metabolic processes* (*GO:0006793,* 40 gene products). There were 15 of those genes associated with *signal transduction by protein phosphorylation (GO:*0023014*)*. Another group of 39 DE-genes is responsible for *cellular response to chemical stimulus (GO:*0070887*)*. In this cluster, 12 DE-genes were associated with the *response to lipids (GO:*0033993*)*. In another group of 38 DE-genes, there was an altered relative abundance of gene transcript as a result of treatment with OXA that are associated with the *regulation of molecular function (GO:*0065009*)*. In this cluster, 20 DE-genes are responsible for *regulation of transferase activity (GO:*0051338*)*. Another group of four genes is associated with the *regulation of receptor activity (GO:*0010469*)*. Furthermore, there is a group of 37 gene products,

Table 1
List of the most significant up- and down-regulated biological process in the orexin A-treated endometrial explants of pigs, in comparison to the non-treated control group; GO: - unique gene onthology identification number; [a]Subset of the process; [b]Part of the process.

GO - Biological Process names			Number of genes	P-value
Up-regulated				
GO:0010033	**Response to organic substance**		**16**	**8,80E-02**
GO:0044248	**Cellular catabolic process**		**15**	**8,41E-03**
[a]GO:0044270	Cellular nitrogen compound catabolic process		5	4,65E-02
GO:0033554	**Cellular response to stress**		**15**	**2,23E-02**
GO:0001819	**Positive regulation of cytokine production**		**5**	**7,58E-02**
[a]GO:0032649	Regulation of interferon-gamma production		3	6,73E-02
GO:1901605	**Alpha-amino acid metabolic process**		**4**	**6,85E-02**
Down-regulated				
GO:0010468	**Regulation of gene expression**		**55**	**5,02E-03**
[a]GO:0010628	Positive regulation of gene expression		35	1,89E-05
[a]GO:0010629	Negative regulation of gene expression		28	5,42E-04
GO:0035556	**Intracellular signal transduction**		**51**	**2,24E-05**
[a]GO:0007166	Cell surface receptor signaling pathway		40	2,78E-03
	[a]GO:0007167	Enzyme linked receptor protein signaling pathway	19	2,24E-03
	[a]GO:1905114	Cell surface receptor signaling pathway involved in cell-cell signaling	9	7,13E-02
[a]GO:0000165	MAPK cascade		15	1,34E-02
[a]GO:0019932	Second-messenger-mediated signaling		11	5,27E-05
[a]GO:0007249	I-kappaB kinase/NF-kappaB signaling		9	8,20E-03
[a]GO:0043491	Protein kinase B signaling		7	4,89E-03
[a]GO:0038061	NIK/NF-kappaB signaling		3	8,31E-02
GO:0009605	**Response to external stimulus**		**49**	**3,63E-08**
[a]GO:0031667	Response to nutrient levels		7	5,14E-02
GO:0009966	**Regulation of signal transduction**		**47**	**2,01E-04**
	[a]GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	7	2,15E-04
[a]GO:1902531	Regulation of intracellular signal transduction		36	4,83E-05
GO:0006793	**Phosphorus metabolic process**		**40**	**4,21E-02**
	[a]GO:0023014	Signal transduction by protein phosphorylation	15	1,45E-02
GO:0070887	**Cellular response to chemical stimulus**		**39**	**3,32E-03**
	[a]GO:0033993	Response to lipid	12	4,98E-02
GO:0065009	**Regulation of molecular function**		**38**	**1,71E-03**
	[a]GO:0051338	Regulation of transferase activity	20	7,93E-04
GO:0010469	**Regulation of receptor activity**		**4**	**9,24E-02**
GO:0006952	**Defense response**		**37**	**4,31E-07**
[a]GO:0006954	Inflammatory response		19	5,02E-05
GO:0072359	**Circulatory system development**		**30**	**2,16E-07**
	[a]GO:0001525	Angiogenesis	12	3,24E-03
GO:0051174	**Regulation of phosphorus metabolic process**		**29**	**1,10E-02**
GO:0042127	**Regulation of cell proliferation**		**28**	**2,11E-03**
GO:0007267	**Cell-cell signaling**		**24**	**3,87E-03**
GO:0046903	**Secretion**		**22**	**7,90E-04**
	[a]GO:0006887	Exocytosis	9	1,52E-02
[a]GO:0048729	Tissue morphogenesis		14	1,70E-02
GO:0002682	**Regulation of immune system process**		**22**	**1,21E-02**
	[a]GO:0002253	Activation of immune response	7	8,64E-02
O:0040007	**Growth**		**21**	**1,28E-03**
[a]GO:0009790	Embryo development		20	1,34E-02

(*continued on next page*)

Table 1 (*continued*)

GO - Biological Process names				Number of genes	P-value
[a]GO:0048598	Embryonic morphogenesis			12	7,53E-02
[a]GO:0001701	In utero embryonic development			11	8,98E-03
GO:0000003	**Reproduction**			**20**	**4,29E-02**
[b]GO:0022414	Reproductive process			20	4,24E-02
	[a]GO:0003006	Developmental process involved in reproduction		15	1,47E-02
		[a]GO:0061458	Reproductive system development	9	8,68E-02
GO:0051093	**Negative regulation of developmental process**			**16**	**8,58E-03**
GO:0098609	**Cell-cell adhesion**			**15**	**9,63E-02**
GO:0051046	**Regulation of secretion**			**17**	**9,61E-04**
	[a]GO:0017157	Regulation of exocytosis		7	9,21E-03
GO:0048771	**Tissue remodeling**			**8**	**2,13E-03**
GO:0031341	**Regulation of cell killing**			**4**	**3,29E-02**
GO:0001906	**Cell killing**			**4**	**9,98E-02**

GO: - unique gene onthology identification number.

[a] Subset of the process.

[b] Part of the process.

with a decreased relative transcript abundance as a result of OXA treatment that are associated with *defence response (GO:*0006952*),* and 19 of these genes are associated with the *inflammatory response (GO:*0006954*)*. There was identification of 30 DE-genes for which there was a decreased abundance of gene transcripts, which are related to the *circulatory system development* processes *(GO:*0072359*)*. In this group, 12 genes are associated with *angiogenesis (GO:*0001525*)*. Another group of 29 and 28 genes for which there was a decreased abundance of gene transcript were modulated by OXA treatment that are associated with *regulation of phosphorus metabolic process (GO:*0051174*)* and *regulation of cell proliferation (GO:*0042127*),* respectively. Furthermore, there was a group of 24 DE-genes for which there was a decreased transcript abundance that are associated with *cell-cell signalling (GO:*0007267*)*. The OXA treatment also affected the abundance of gene transcripts for a group of 22 genes that are associated with *secretion (GO:*0046903*)*. In this cluster, 14 DE-genes are involved in *tissue morphogenesis (GO:*0048729*)* and seven with *exocytosis (GO:*0006887*)*. The treatment with OXA also affected the abundance of gene transcripts for 22 DE-genes responsible for *regulation of immune system processes (GO:*0002682*)*. In this group, two genes were identified for which products are associated with the *activation of immune response (GO:*0002253*)*. The OXA treatment also affected the relative abundance of transcripts for 21 genes encoding factors associated with *growth (GO:*0040007*)*. In this cluster, OXA modulated *embryo development* (*GO:*0009790; 20 gene products*), embryonic morphogenesis* (*GO:*0048598; 12 gene products) and *in utero embryonic development* (*GO:*0001701; 11 gene products). Furthermore, there was a group of 20 DE-genes, modulated by OXA treatment, that were associated with *reproduction (GO:*0000003*)*. In this group, the hormone influenced genes involved in: *reproductive processes* (*GO:*0022414; 20 gene products), *developmental processes involved in reproduction* (*GO:*0003006; 15 gene products) and *reproductive system development* (*GO:*0061458; nine gene products). Other biological processes modulated by OXA include *negative regulation of developmental process* (*GO:*0051093; 16 gene products) and *cell-cell adhesion* (*GO:*0098609; 15 gene products). Another group of 17 gene products, modulated by OXA, is associated with *regulation of secretion (GO:*0051046*)*. In this cluster, seven DE-genes are involved in the *regulation of exocytosis (GO:*0017157*)*. The DE-genes of the remaining three groups were down-regulated as a result of treatment with OXA, and include *tissue remodelling* (*GO:*0048771; eight gene products), *regulation of cell killing* (*GO:*0031341; four gene products) and *cell killing* (*GO:*0001906; four gene products).

3.3. Biological pathways

Using the KEGG database, 26 biological pathways were generated (Table 2.). Pathways with the largest number of DE-genes were associated with *cancer* and *endocytosis.* There were 22 and 14 DE-genes associated with this pathways, respectively. The other

Table 2
Analysis of pathways where there was a differential abundance of gene transcripts in endometrial tissues of pigs treated with orexin A.

KEGG pathway analysis			
KEGG pathway analysis name	Number of genes	P-value	Altered genes
Pathways in cancer	22	8,20E-04	CEBPA. GNA13. DVL3. BCR. EPAS1. MAP2K1. MITF. SPI1. FOXO1. NFKBIA. SMAD3. STAT1. APPL1. MMP1. EDNRA. WNT2. CBLB. CDKN2B. CXCR4. NCOA4.
Endocytosis	14	8,90E-03	CBLB. SMAP2. USP8. ARF1. CXCR4. FOLR1. LOC100522401. SMAD3. HSP70. NEDD4L. ARFGEF1. HSPA8. DNM1
HTLV-I infection	12	4,10E-02	WNT2. DVL3. CDKN2B. ETS1. MAP3K1. SPI1. SMAD3. NFKBIA. ANAPC7. NFATC2. ATM. APC
cAMP signaling pathway	10	4,40E-02	EDNRA. PPARA. MAP2K1. PLN. NFKBIA. GNAS. PDE4D. SOX9. ADORA1. SLC9A1
AMPK signaling pathway	9	4,60E-03	LEP. CD36. PPP2R5A. ACACA. RAB14. FOXO1. SCD5. CCNA1. PPP2R2 A
Signaling pathways regulating pluripotency of stem cells	9	9,00E-03	WNT2. DVL3. ACVR2 A. MAP2K1. TBX3. POU5F1. SMAD3. ID4. APC
Osteoclast differentiation	9	1,00E-02	CYLD. MAP2K1. MITF. SPI1. ACP5. NFKBIA. NFATC2. STAT1. SYK
Epstein-Barr virus infection	9	7,20E-02	CSNK2 A2. FGR. SPI1. NFKBIA. POLR3A. HSP70. CCNA1. HSPA8. SYK
cGMP-PKG signaling pathway	8	8,60E-02	GNA13. EDNRA. MAP2K1. ATP2 A2. PLN. PRKCE. NFATC2. ADORA1
Influenza A	8	8,60E-02	NUP98. MAP2K1. NFKBIA. HSP70. OAS2. STAT1. CASP1. HSPA8
Legionellosis	7	1,40E-03	ARF1. NFKBIA. HSP70. TLR5. HSPD1. CASP1. HSPA8
mRNA surveillance pathway	7	6,90E-03	SYMPK. PPP2R5A. ACIN1. CSTF2T. PABPC1L. WDR33. PPP2R2 A
B cell receptor signaling pathway	6	1,30E-02	MAP2K1. NFKBIA. PIK3AP1. CD79 A. NFATC2. SYK
RNA degradation	6	1,40E-02	PAN2. CNOT9. BTG1. ENO2. HSPD1. PABPC1L
Melanogenesis	6	4,10E-02	WNT2. DVL3. MAP2K1. MITF. GNAS. ASIP
Estrogen signaling pathway	6	4,70E-02	MAP2K1. FKBP5. ESR1. GNAS. HSP70. HSPA8
Insulin resistance	6	8,60E-02	PPARA. CD36. PPP1R3B. FOXO1. NFKBIA. PRKCE
Sphingolipid signaling pathway	6	8,60E-02	GNA13. MAP2K1. PPP2R5A. PRKCE. ADORA1. PPP2R2 A
Thyroid hormone signaling pathway	6	8,80E-02	MAP2K1. PLN. ESR1. FOXO1. STAT1. SLC9A1
PPAR signaling pathway	5	3,60E-02	PPARA. CD36. FABP1. SCD5. MMP1
Bile secretion	5	3,80E-02	SLC9A3. GNAS. ABCC2. SLC9A1. ABCG2
RIG-I-like receptor signaling pathway	5	3,80E-02	CYLD. CASP10. SIKE1. MAP3K1. NFKBIA
Gap junction	5	9,20E-02	MAP2K1. GJA1. GNAS. PDGFC. HTR2B
NF-kappa B signaling pathway	5	9,60E-02	CSNK2 A2. NFKBIA. ATM. TAB3. SYK
Inflammatory bowel disease (IBD)	4	8,50E-02	IL12RB2. SMAD3. TLR5. STAT1
Endocrine and other factor-regulated calcium reabsorption	3	1,00E-01	ESR1. GNAS. DNM1

pathways that were affected by treatment with OXA were detected using the DAVID technique and these were: *human T-lymphotropic virus (HTLV-I) infection* (12 DE-genes), *cyclic AMP* (*cAMP) signalling pathway* (10 DE-genes), *AMP-activated protein kinase (AMPK) signalling pathway* (nine DE-genes*), signalling pathways regulating pluripotency of stem cells* (nine DE-genes), *osteoclast differentiation* (nine DE-genes), *Epstein-Barr virus infection* (nine DE-genes), *cGMP-dependent protein kinase* (*cGMP-PKG) signalling pathway* (eight DE-genes), *influenza A* (eight DE-genes), *legionellosis* (seven DE-genes), *mRNA surveillance pathway* (seven DE-genes), *B cell receptor signalling pathway* (six DE-genes), *RNA degradation* (six DE-genes), *melanogenesis* (six DE-genes), *oestrogen signalling pathway* (six DE-genes), insulin resistance (six DE-genes), *sphingolipid signalling pathway* (six DE-genes), *thyroid hormone signalling pathway* (six DE-genes), *PPAR signalling pathway* (five DE-genes), *bile secretion* (five DE-genes), *RIG-I-like receptor signalling pathway* (five DE-genes), *gap junction* (five DE-genes), *NF-kappa B signalling pathway* (five DE-genes), *inflammatory bowel disease (IBD)* (four DE-genes), *and endocrine and other factor-regulated calcium reabsorption* (three DE-genes).

3.4. Network among genes with differential abundances of transcripts

During the interaction analysis between the genes associated with the synthesis, activity and metabolism of hormones and other biological substances, these were all associated through one network (Fig. 1.). The 96 interactions were associated with gene co-expressions. In 156 cases, selected gene expression was co-localised. Genetic interactions were present in 22 cases. Nine interactions were associated with shared protein domains. There was not detection of any physical interactions between selected genes. Supplementary file 5 contains a complete list of gene interactions.

3.5. Real-time PCR validations

To validate the microarray analysis results, the relative abundance of transcripts for 11 genes with a fold change of greater than 1.2 has been analysed using the qPCR method. All changes in relative abundance of gene transcripts determined in the microarray analysis has been confirmed by real-time analysis (Fig. 2.). Data are presented as means ± S.E.M. from four different observations ($n = 4$).

4. Discussion

To the best of our knowledge, this is the first study to analyse the influence of OXA on global gene expression in the mammalian reproductive tract. The aim of the study was to determine the effects of OXA on the transcriptomic profile in *in vitro* cultured endometrial tissue explants from early-pregnant gilts, and to identify the changes in gene transcript abundance when there was treatment with OXA. The 4 × 44 Porcine (V2) two-colour gene expression microarray (Agilent, USA) was used. Based on previous reports exploring this microarray technology (Szeszko et al., 2016; Li et al., 2017; Zglejc et al., 2018), only DE-genes with FC greater than 1.2 were included in the bioinformatic analysis. The list of DE-genes with FC greater than 1.2 was used to analyse specific gene ontologies, biological pathways and interaction networks. In the present study, 1,242 DE-genes were identified, including 1,104 genes with an FC greater than 1.2. In the group of genes for which these FC criteria were met, 457 had an increased and 647 had a decreased abundance of gene transcript in the animals treated with OXA relative to the control group. The microarray analysis of the endometrial tissue of pigs was conducted during early pregnancy, on days 15 to 16 of gestation, which is a period where there are dynamic uterine changes that are important for the maintenance of pregnancy and embryo survival. In pigs, days 15 to 16 of gestation is when there is the beginning of implantation, dynamic tissue remodelling of endometrial tissue, embryo localisation and development of trophoblastic tissues which are involved in implantation of the embryo in the uterus. Successful implantation is a result of many processes which involve many genes and the resulting products and which lead to the development of a uterine capacity that is conducive for embryo/foetal development (for more see: Bazer and Johnson, 2014).

The expression of the genes which result in the production of proteins for the orexin system (orexins and the respective receptors) in female reproductive structures has been confirmed in many species, including the uteri, trophoblasts and conceptuses of pigs (Nitkiewicz et al., 2012; Smolinska et al., 2015), and in human, dog and cat placentae (Nakabayashi et al., 2003; Dall'Aglio et al., 2012 2014). There, however, is a general scarcity of data concerning the effect of orexins during the early pregnancy of pigs. Results of previous studies indicate that both OXA and OXB affect the endometrial and myometrial expression of genes encoding for steroidogenic enzymes as well as the secretion of steroid hormones during early gestation. The effect of both OXA and OXB was determined by assessing changes in hormone concentration during gestation with results indicating the local hormonal milieu is changed during gestation (Kiezun et al., 2017; Kaminski et al., 2018). Even though there are these effects of orexins on uterine functions, very little is known about the overall effect on the processes that take place in a uterus of pregnant pigs. The results of the transcriptomic analysis of the effect of the OXA on global gene expression in the endometrium of pregnant gilts could provide valuable insights, which, in the future may be applied in animal breeding for purposes of increasing litter size and decreasing pregnancy losses. There is general agreement that there is death of as many as 40% of pig embryos in early pregnancy, between days 12 and 18 of gestation. The gestational time-period studied in the present study is the critical period for embryo survival and further development. Understanding changes which occur in the pregnant uterus during this period when there are effects of such an important metabolic hormone as OXA would be helpful in a proper optimisation of animal breeding and nutrition.

The results of gene ontology analysis indicate that OXA has a modulatory effect on numerous genes involved in *intracellular signal transduction (GO:*0035556*) and regulation of signal transduction (GO:*0009966) in the pig endometrium. A group of 15 genes in these clusters is associated with the *mitogen-activated protein kinases (MAPK) cascade,* and seven genes are involved in the *regulation of*

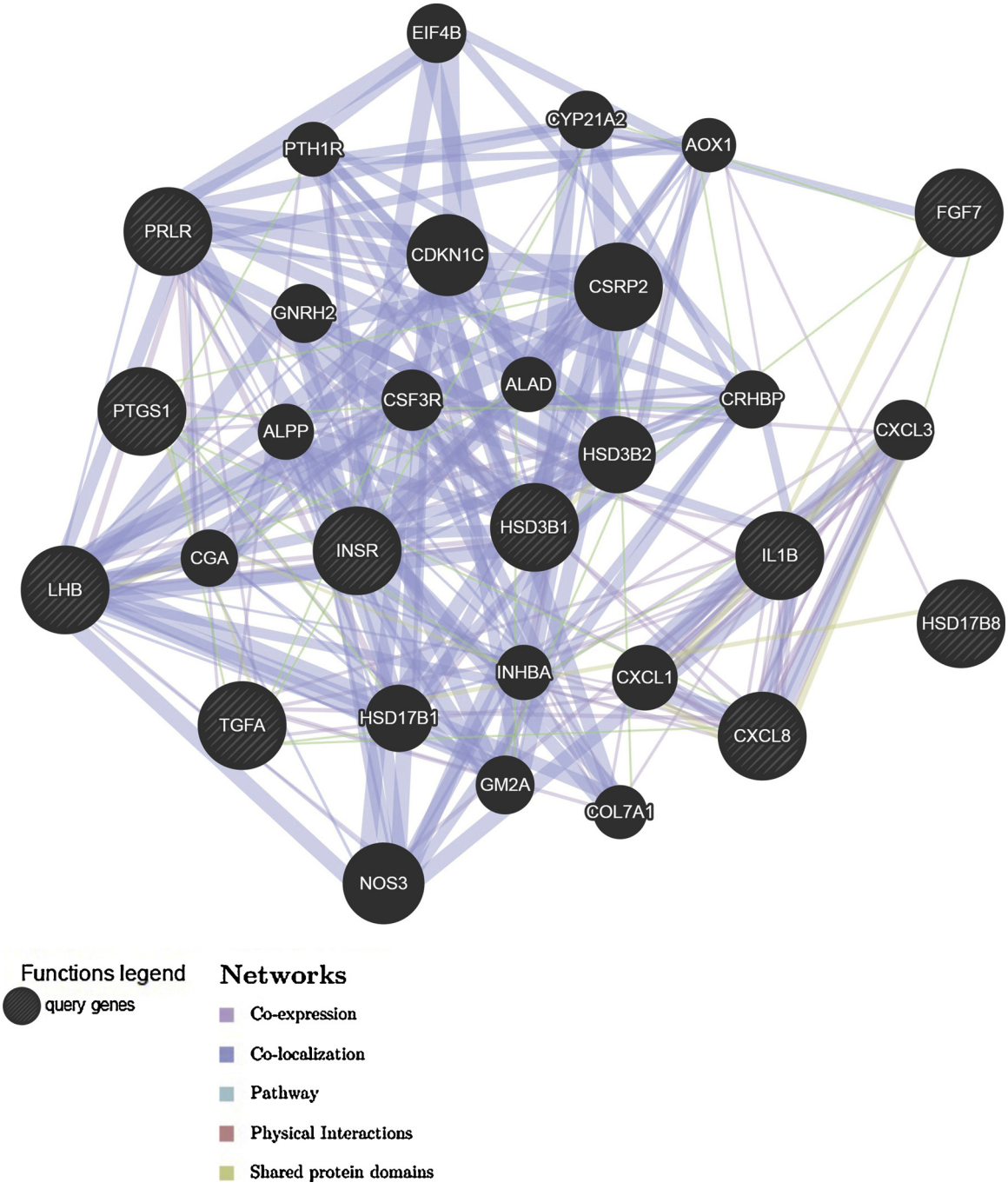

Fig. 1. Gene interaction network developed in GeneMania for selected genes; Colour of line connecting the genes denotes the type of interaction (refer to the key).

phosphatidylinositol 3-kinase signalling (GO:0014066*)*. The *MAPK/ extracellular signal–regulated kinase (ERK1/2) (MAPK/ERK1/2) pathways and PI3K/protein kinase B (AKT) (PI3K/AKT)* pathways are essential for mediating the regulation of trophoblast growth and attachment to the uterus (McKinnon et al., 2001). In humans, prolonged (7–10 days) fasting resulted in an increase in serum OXA concentrations (Komaki et al., 2001). In rats, 48 h of fasting induced an increase in both *PPO* gene expression and OXA protein concentration (Cai et al., 1999; Mondal et al., 1999). The OXA-dependent decrease in the expression of genes implicated in the MAPK cascade could be associated with a lesser implantation ratio and, consequently, with decreased expenditure of maternal energy during embryo development. This mechanism could be a part of wider control system which prevents excessive energy expenditure when food is scarce. The results of the gene ontology analysis indicate that OXA inhibits the expression of genes controlling growth

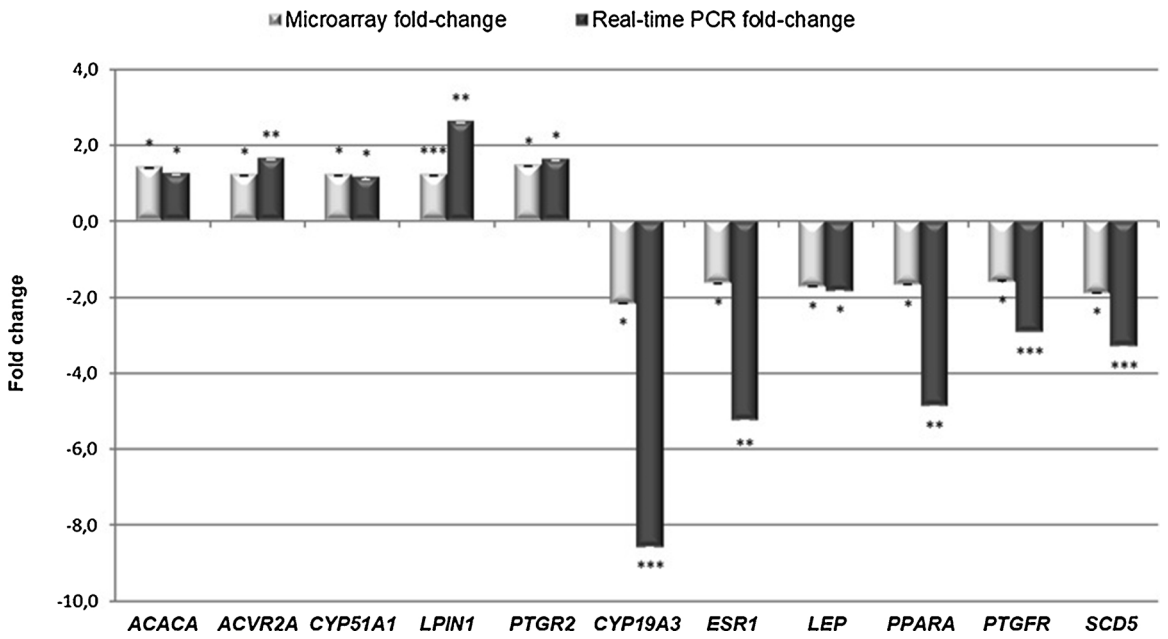

Fig. 2. qPCR validation of the microarray experiment; Light grey bars represent fold changes for microarray data; dark grey bars represent fold changes for qPCR data; Data presented as means ± SEM from four different observations; *P < 0.05; **P < 0.01; ***P < 0.001.

(GO:0040007), reproduction (GO:0000003) and regulation of cell proliferation (GO:0042127), which provides support for the research hypothesis of the present study. These results indicate that OXA, the most important hormone responsible for controlling energy metabolism, could be involved in the regulation of litter size during implantation, depending on the metabolic and nutritional status of the dam. There may be similar effects when there is starvation or nutrition deficiency that inhibits reproductive functions of gilts by compromising the processes of implantation.

The results of the gene ontology analysis indicate that OXA suppressed the expression of genes involved in the inflammatory response (GO:0006954), cytokine production (GO:0001816), regulation of cytokine production (GO:0001817), regulation of cytokine secretion (GO:0050707) and cytokine response (GO:0034097). In laboratory animals, pro-inflammatory factors such as cytokines were responsible for a decrease in appetite and food intake. Treatments with interleukin 1β (IL1β) and lipopolysaccharide (LPS), which induce the release of pro-inflammatory cytokines, reduced both appetite (LPS and IL1β) and food intake (IL1β) (Bret-Dibat et al., 1995; Langhans et al., 1993). Furthermore, central administration of interleukin-6 (IL6) and tumour necrosis factor alpha (TNFα) induced a decrease in food intake (Kapás and Krueger (1992); Plata-Salamán et al., 1996). The expression of the OXA gene was also decreased during acute inflammation (Yan et al., 2005). Function of the orexin system is impaired by TNFα resulting in a lesser relative abundance of *PPO* and *OX2R* mRNA after treatment with TNFα (Zhan et al., 2011). It, however, has been suggested that orexins could have anti-inflammatory effects in neuroinflammatory diseases and oxidative stress caused by cerebral ischemia (Xiong et al., 2013; Clark and Vissel (2014)). In addition, OXA regulates infection-induced inflammation by modulating TNF-α production in microglia of mice (Xiong et al., 2013). During implantation, pig conceptuses produce various compounds which modulate the maternal immune system. Semi-allogenic conceptuses induce the expression of many genes regulating the inflammatory response in the uterus (Bazer and Johnson, 2014; Geisert et al., 2012, 2014). During the peri-implantation period of pigs, the endometrial tissue secretes various cytokines and growth factors, including IL-6, epidermal growth factor (EGF), transforming growth factor beta (TGFB) and leukaemia inhibitory factor (LIF) (Vaughan et al., 1992; Anegon et al., 1994; Jaeger et al., 2005). The cytokines released by the endometrium stimulate trophectoderm proliferation and migration *via* the PI3K/AKT and ERK1/2/MAPK signalling pathways (Jeong et al., 2013, 2014). Furthermore, the endometrial activation of inflammatory factors and pathways enhance uterine receptivity for conceptus implantation (Warning et al., 2010). The inflammatory response and cytokine activity, however, is tightly controlled to prevent the rejection of semi-allogenic conceptuses. In the present study, OXA may have affected the controlled spread of inflammation by inhibiting the genes that encode the production of cytokines and inflammation.

The results from analysis of DE-genes indicate that OXA inhibited the expression of leptin (*LEP*) gene. Leptin as well as adiponectin, visfatin, omentin and chemerin are adipocyte-derived metabolic hormones with pleiotropic effects. The results of the gene ontology analysis indicate that leptin frequently participates in the processes regulated by OXA. Initially, LEP was regarded as a hormone that was responsible exclusively for controlling feed intake (Considine et al., 1996); however, there is evidence to indicate that it exerts pleiotropic effects on various processes, including hemopoiesis and reproductive functions (Bennett et al., 1996; Barash et al., 1996). In pigs, the LEP protein and/or the expression of LEP receptor genes has been confirmed to occur in structures responsible for reproductive functions, including the ovaries (Bogacka et al., 2006; Smolinska et al., 2007a), hypothalamus and pituitary gland (Bogacka et al., 2006; Kaminski et al., 2006; Smolinska et al., 2004; Siawrys et al., 2009) uterus (Bogacka et al., 2006; Smolinska et al., 2007b), as well as in trophoblasts (Smolinska et al. 2007b), during the oestrous cycle and early gestation. In mice,

intrauterine injection of a leptin antagonist resulted in embryo implantation failure (Ramos et al., 2005). Furthermore, leptin enhances the development of pig embryos (Craig et al., 2004). Leptin and OXA have the opposite effects on the food intake, and the plasma concentrations of these two hormones are inversely correlated (Shiraishi et al., 2000; Komaki et al., 2001). In other studies, OXA activated leptin-responsive neurons in the arcuate nucleus of rats (Rauch et al., 2000) whereas leptin inhibited a fasting-induced increase in the expression of the *PPO* gene in the hypothalamus of rats (López et al., 2000). These findings indicate that LEP and OXA are part of a mutual control system regulating energy expenditure. This indicates that when food intake is limited, there is an enhanced function of OXA relative to that of LEP and a minimisation of the beneficial effects of LEP on embryo development to reduce the expenditure of energy during reproductive processes.

5. Conclusion

This is the first study where there was investigation of the effects of OXA on global gene expression in the pig endometrium during implantation. The present findings indicate that OXA, one of key metabolic regulators, affects the abundance of a large number of gene transcripts and processes in the endometrium of pregnant pigs. The OXA protein, therefore, could be an important regulator of implantation, depending on maternal nutritional status.

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Conflict of interest

None.

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Raw data files from the microarray experiments have been uploaded on the Gene Expression Omnibus (GEO) server (https://www.ncbi.nlm.nih.gov/geo/; accession number: GSE115195).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.anireprosci.2018.11.008.

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