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Transcriptomic profile of anterior pituitary cells of pigs is affected by adiponectin

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ABSTRACT

Adiponectin is thought to be involved in the regulation of metabolic homeostasis and reproductive processes. It also has an important role in the modulation of female reproductive functions, both directly and by affecting the secretory functions of the hypothalamic-pituitary-gonadal axis. The main aim of this study was to determine the effect of adiponectin on global gene expression and on differentially expressed genes (DE-genes) regulated by adiponectin in anterior pituitary (AP) cells of pigs. The changes in the transcriptomic profile of AP cells of pigs were examined using the Porcine (V2) two-colour gene expression microarray, 4 × 44. An analysis of data from the microarray experiment indicated there were 716 DE-genes. A total of 466 genes (220 up-regulated and 246 down-regulated) with fold change greater than 1.2 ($P < 0.05$) were subsequently selected for further analysis. Gene ontology was analysed based on a list of DE-genes. A list of biological processes was generated for both up-regulated and down-regulated DE-genes. The products of up-regulated DE-genes were involved in 60 biological processes, whereas for down-regulated products there were 18 processes. An analysis of the interactions between DE-genes indicated that adiponectin interacted with genes that potentially encode intracellular signalling pathways and factors which regulate reproductive functions. Furthermore, nine genes were selected from the list of DE-genes to confirm microarray results by quantitative PCR. The results enhance the knowledge about adiponectin's role in the pituitary functions of pigs and provide valuable insights for further studies into adiponectin's mechanism of action in the pituitary.

1. Introduction

The correlation between energy metabolism and reproduction in animals has been studied for many years. There is evidence to indicate that metabolic and reproductive systems are co-controlled by specific hormones. The effects of leptin, ghrelin and orexins on the metabolic status and reproductive function have been thoroughly documented (Kaminski et al., 2006; Rak and Gregoraszczuk, 2008; Smolinska et al., 2009, 2015; Kiezun et al., 2017). There are also results that indicate adiponectin also regulates metabolic homeostasis and reproduction.

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Adiponectin is one of the most widely occurring proteins in mammalian blood serum (2–25 μg/ml; Kadowaki and Yamauchi, 2005). A primary factor regulating the physiological effects of adiponectin are the two types of receptors: adiponectin receptor type 1 (AdipoR1) and type 2 (AdipoR2). Adiponectin receptors (AdipoRs) are widely distributed in peripheral tissues and organs, and the hormone has pleiotropic effects on metabolism. In the liver, adiponectin promotes glucose transport, inhibits gluconeogenesis, activates oxidation of fatty acids and enhances insulin sensitivity promoting phosphorylation of the insulin receptor (Wu et al., 2003; Kadowaki and Yamauchi, 2005; Ye and Scherer, 2013). In white adipose tissue, adiponectin promotes basal and insulin-stimulated glucose uptake and regulates lipid metabolism by inhibiting lipolysis (Wu et al., 2003; Qi et al., 2004; Wedellová et al., 2011). The elements of the adiponectin system are present in the hypothalamic-pituitary-gonadal (HPG) axis of humans, pigs, rats, cows and chickens (Ledoux et al., 2006; Chabrolle et al., 2007a, 2007b, 2009; Mailard et al., 2010; Kiezun et al., 2013; Kaminski et al., 2014; Maleszka et al., 2014a). The distribution of AdipoRs in human, pig, rat, mouse and chicken hypothalami and pituitaries implies that adiponectin could modulate the endocrine functions of the central and, indirectly, the peripheral branches of hormonal axes, including the HPG axis (Spranger et al., 2006; Psilopanagioti et al., 2009; Steyn et al., 2009; Kiezun et al., 2013). Adiponectin also appears to have an important role in auto/paracrine control of pituitary somatotrophs and gonadotrophs (Rodriguez-Pacheco et al., 2007). In the study by Rodriguez-Pacheco et al. (2007) with rats, adiponectin had an effect on basal and ghrelin-, GHRH- and GnRH-stimulated GH and LH release. It appears as though this effect occurs through adiponectin actions on cellular receptors. Furthermore, results from *in vitro* studies indicate basal secretion of follicle stimulation hormone (FSH) was modulated by adiponectin during the oestrous cycle; however, adiponectin did not affect the basal secretion of LH in AP cells of pigs. Adiponectin administration also affected GnRH- and/or insulin-induced LH and FSH release in a manner dependent on the phase of the oestrous cycle (Kiezun et al., 2014).

There, however, is a general scarcity of data relating to adiponectin's effects on global gene expression in the HPG axis, including the anterior pituitary. It is, therefore, hypothesised that this hormone could have an important function in the regulation of AP cells. The aim of the present study was to assess differentially expressed genes in pituitary cells of pigs after treatment with adiponectin.

2. Materials and methods

2.1. Experimental animals

All experiments were conducted in accordance with the principles of the ethical standards set forth by the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn (decision No. 91/2011/DTN). The study was performed on mature gilts (Large White × Polish Landrace) aged 7–8 months, with body weights of 120–130 kg, housed at a private farm, as described previously (Maleszka et al., 2014a). Pituitaries ($n = 4$) were collected in the mid-luteal phase (day 10–12) of the oestrous cycle. The mid-luteal phase is characterised as the time when there is maximal CL function (production of significant amounts of progesterone). During this phase, there was an effect of adiponectin on LH and FSH release from anterior pituitary cells of pigs (Kiezun et al., 2014). Gilts were observed daily for oestrus behaviour in the presence of an intact boar. The onset of the second oestrus was marked as day 0 of the oestrous cycle. The mid-luteal phase of the oestrous cycle was also confirmed based on presence and morphology of the CL (colour, the lack of blood clot inside the CL and the morphology of the CL vasculatory; Akins and Morrissette, 1968). The animals were stunned using electricity and exsanguinated in specialised slaughterhouse for collection of experimental tissues. Immediately after slaughter, the pituitaries were removed, placed on ice and separated into anterior (AP) and posterior (NP) parts. Isolated AP lobes for cell cultures were placed in chilled Dulbecco's modified Eagle's medium (DMEM) with 0.1% BSA, and transported to the laboratory.

2.2. Isolation and in vitro cultures of anterior pituitary cells

The procedure of AP tissue dissociation, cell preparation, and *in vitro* culture was previously described in detail by Kiezun et al. (2014). Anterior pituitary lobes were washed with fresh DMEM, minced into 1- to 2-mm pieces and trypsinised with 0.25% trypsin (7–8 times, 10 min., 37 °C). At least three to four pituitaries were pooled to achieve the apropriate number of cells for each biological replicate. Dispersed pituitary cells were transferred to plastic tubes, repeatedly centrifuged at 1200 *g* for 10 min, and washed with DMEM three times. After the final centrifugation, pituitary cells were filtered through a nylon filter (60 μm mesh) and counted in a hemocytometer. Cell viability (90%–97%) was determined by trypan blue dye exclusion. Pituitary cells were then re-suspended in McCoy's 5A medium with 10% horse serum, 2.5% foetal calf serum (FCS), 240 U/ml nystatin and 100 μg/ml gentamycin, supplemented with 0.1% vitamins and 0.01% nonessential amino acids. Dispersed cell suspension (1 ml; 2×10^6 cells/ml) was transferred to each 6-well culture plate. The cells were allowed to attach for 48 h (37 °C, 95% O_2 and 5% CO_2). After pre-culture, the cells were incubated for 24 h (37 °C, 95% O_2 and 5% CO_2) in 1 ml of McCoy's 5A medium (without serum) containing bacitracin (2×10^{-5} M) with or without (control culture) recombinant human adiponectin (BioVendor, USA) at a concentration of 10 μg/ml. The adiponectin dose was selected based on our earlier experiments (Maleszka et al., 2014a, 2014b), and is equivalent to the physiological concentration of this hormone (Maleszka et al., 2014b). All incubations were performed in triplicate (three wells for control culture, and three wells with adiponectin per *n,* respectively). Before the RNA isolation all cells from each of the three wells were pooled. The AlamarBlue test (MP Biomedicals, USA) indicated that adiponectin did not affect the viability of the cultured AP cells. Cell pre-incubation and incubation times were modified (48 h + 24 h) in the AlamarBlue test, as described previously (Szeszko et al., 2016).

2.3. Total RNA isolation and microarray analysis

Total RNA isolation, quality control of the isolated material and the microarray analysis were performed using the procedures described by Szeszko et al. (2016). Total RNA from each AP cell culture was isolated with the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Additionally, RNA was purified using on-column digestion of DNA with DNase I (Qiagen, USA). The concentration and purity of the isolated RNA were determined by spectrophotometry (Infinite 200 PRO, Tecan Group, Switzerland). The RNA integrity number (RIN) was evaluated in the Agilent Bioanalyser 2100 (Agilent Technology, USA). The RNAs with RIN of greater than eight were used for microarray and quantitative real-time PCR (qPCR) validation experiments. Total RNA was amplified and labelled using the LowInput Quick Amp Two-Colour Labelling kit (Agilent Technologies, USA) according to the manufacturer's instructions. The sample and control RNAs were labelled with Cyanine-5 (Cy-5) and Cyanine-3 (Cy-3). Dual-labelled cRNA was fragmented, mixed with the hybridisation buffer and added to the Porcine (V2) Gene Expression Microarray 4 × 44 (Agilent Technologies, USA) according to the manufacturer's instructions. Each array ($n = 4$, one slide) consisted of two differentially labelled cRNA samples (obtained from adiponectin-treated and control samples) in a balanced block design with dye-swaps. Hybridisation was conducted in a hybridisation oven (60 °C, 17 h, Agilent Technologies, USA). After hybridisation, the microarrays were evaluated at 5 µm resolution and a TIFF image was generated. In the next step of the microarray analysis, the TIFF image was uploaded to the Feature Extraction (FE) Software (Agilent Technologies, USA) to extract feature data, perform detailed analyses, distinguish the features from the background and normalise dyes (linear and LOWESS). 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: GSE122311).

2.4. Bioinformatics analysis

The extracted feature data were uploaded to the GeneSpring GX 12 software (Agilent Technologies, USA) to identify differentially expressed (DE) genes in adiponectin-treated and control samples. The FC cut-off for significant DE-transcripts was set at > 1.2 for up- and down-regulated DE-genes at a *P*-value < 0.05. The 1.2 FC cut-off was chosen based on previous results from previous studies (Szeszko et al., 2016; Kang et al., 2017; Li et al., 2017; Dobrzyn et al., 2018). The FC was calculated based on the mean extent of gene expression of adiponectin-treated/control samples in four replicates. The list of DE-genes was manually enriched using the Basic Local Alignment Search Tool (BLAST) by aligning unknown gene probe sequences with the entire porcine transcriptome deposited in the database. For DE-genes with replicated probes, the mean FC value was calculated for all probes in the array. Significant differences between the normalised fluorescence intensity of adiponectin-treated and control samples were determined using the Student's t-test.

2.4.1. Gene Ontology (GO) analysis

The list of differentially expressed genes (DE-genes) was uploaded to the Database for Annotation, Visualisation and Integrated Discovery version 6.8 (DAVID, http://david.abcc.ncifcrf.gov) to explore the functional class of genes based on evolutionary relationship (Huang et al., 2009). The gene ontology (GO) analysis was limited to *Sus scrofa*, and genome of this species was used as the background. Significance levels were calculated in a modified Fisher's exact test ($P < 0.1$).

2.4.2. Biological pathways analysis

The biological pathways enriched from the gene list were also generated in the DAVID application. Based on the code of every individual gene, the program computes the overrepresentation value for each pathway represented in the Kyoto Encyclopaedia of Genes and classifies DE-transcripts into groups with different biological functions and pathways. Significance levels were calculated using a modified Fisher's exact test.

2.4.3. A network of interactions between differentially expressed genes

Nine genes were selected from the list of differentially expressed genes with FC > 1.2 to develop a network of interactions: dihydropyrimidinase (*DPYS*), gamma-aminobutyric acid type B receptor subunit 2 (*GABBR2*), growth factor receptor-bound protein 14 (*GRB14*), natriuretic peptide receptor 2 (*NPR2*), peroxisome proliferator-activated receptor alpha (*PPARA*), prostaglandin E receptor 4 (*PTGER4*), solute carrier family 5 member 1 (*SLC5A1*), tumour necrosis factor superfamily member 13b (*TNFSF13B*) and tryptophanyl-tRNA synthetase (*WARS*). These genes were selected based on the likelihood of numerous possible interactions. Products of the selected genes are involved in various processes that affect the function or metabolism of reproductive hormones.

The gene interaction network was created with the GeneMania Prediction Server (Warde-Farley et al., 2010). GeneMania networks were developed based on known gene interactions such as co-expression, co-localisation, genetic interactions, signalling pathways, physical interactions and shared protein domains.

2.5. Real-time PCR validations

The nine genes used in the interaction network analysis were selected to confirm microarray results by qPCR. Quantitative PCR was preceded by complementary DNA (cDNA) synthesis using the same RNA ($n = 4$ for both adiponectin-treated and control probes) as in the microarray analysis. One µg of each total RNA sample was transcribed with the Omniscript RT Kit (Qiagen, USA), a mix of dNTPs and 0.5 µg oligo$(dT)_{15}$ (Roche, Germany) in a total volume of 20 µL. The reaction was conducted at 37 °C for 1 h and was

terminated by incubation at 93 °C for 5 min. Quantitative PCR was performed in duplicate for each sample using the 7300 Real-Time PCR system and the Power SYBR® Green PCR Master Mix (Life Technologies, USA) as described previously by Szeszko et al. (2016). The sequences of the selected primer pairs (forward and reverse), qPCR conditions and primer concentrations are presented in Supplementary file 1 (SF.1.). Amplification specificity was tested by non-template controls and dissociation curve analysis of the amplified products in the final stage of PCR for each reaction. The Ct values for all non-template controls were below the detection threshold. Extent of gene expression calculated by the comparative cycle threshold method (ΔΔCT) and normalised using the geometric mean of two reference genes: β-actin (*ACTB*) and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The expression of *ACTB* and *GAPDH* genes did not differ significantly between adiponectin-treated and control samples. Data are presented as means ± S.E.M. from four different observations. Differences between treated sample and control were analysed using a one-way ANOVA followed by use of a least significant differences (LSD) *post hoc* test. Statistical analyses were performed using STATISTICA Software (StatSoft Inc., Tulsa, USA). Differences were regarded as statistically significant at $P < 0.05$.

3. Results

3.1. Microarray data analysis

3.1.1. Description of differentially expressed genes

A complete list of genes that were differentially expressed in control cells and cells treated with studied hormone (adiponectin) is presented in Supplementary file 2 (SF.2.). The table includes the name of the probe set, gene ID, FC, *P*-value, direction of change (up or down), function and accession number. In the group of 716 differentially expressed genes that were selected with the use of GeneSpring software, 466 were characterised by an FC of greater than 1.2 ($P < 0.05$). The expression of 220 genes increased and the expression of 246 genes decreased in AP cells treated with adiponectin.

3.1.2. Gene Ontology (GO) analysis

The DE-genes were functionally classified using the DAVID bioinformatics technique. Three GO categories were created: biological process (BP), molecular function (MF) and cell component (CC). Biological process ontology analyses describe a series of events that occurs as a result of one or more ordered assemblies of molecular functions associated with DE-genes. Molecular functions are related to activities that occur at the molecular level, including catalytic and binding functions. Cellular component ontology refers to the location of gene products at the subcellular level and at macromolecular complexes. The results for the entire DE-gene list (up- and down-regulated; modified Fisher's exact test, $P < 0.1$) are shown in Supplementary file 3 (SF.3.). The pituitary gland has a major endocrine function in females; therefore, the biological process category was most significant for research in the present study. A list of biological processes associated with up-regulated and down-regulated DE-genes was also generated (Supplementary file SF.4.). Based on the list, there was selection of the 50 most important processes for the anterior pituitary gland, including 32 and 18 processes related to up- and down-regulated DE-genes, respectively (Table 1). The columns in the table indicate the direction of expression of the group of genes (up or down), the name and accession number of the BP, the number of counts, *P*-value and the names of genes in the assigned BP.

The results of the GO analysis indicated there were changes in the expression of numerous genes that are involved in different signal transduction pathways in AP cells that are affected by adiponectin. A number of up-regulated DE-gene groups in the *MAPK cascade (GO:*0000165*)* and *Phosphatidylinositol 3-kinase signaling (GO:*0014065*)* was identified. There was a group of ten gene products that modulate *regulation of intracellular signal transduction (GO:*1902531*)*, including three products that affect the *regulation of phosphatidylinositol 3-kinase signalling (GO:*0014066*)*. Other biological processes that are modulated by adiponectin and are related to signal transduction include five DE-genes which function in the *positive regulation of the MAPK cascade (GO:*0043410*)*. In this group, three and two gene products are associated with: *positive regulation of ERK1 and ERK2 cascades* (*GO:*0070374) and *activation of MAPKK activity* (*GO:*0000186), respectively. These results indicate adiponectin could affect the signal transduction pathways in AP cells by modulating the expression of DE-genes associated with these GO terms. In regards to adiponectin's effect on pituitary secretory functions, there was identification of three processes related to up-regulated DE-genes and one process related to down-regulated DE-genes. The results of the analysis, based on the list of up-regulated DE-genes, indicate there is a group of four gene products associated with GO terms that affect the *positive regulation of cytokine production (GO:*0001819*)*, three gene products that affect the *positive regulation of cytokine secretion (GO:*0050715*)*, and three gene products that affect the *positive regulation of protein secretion (GO:*0050714*)*. There was also identification of a group of products of down-regulated DE-genes that modulate the *positive regulation of secretion* (three DE-genes; *GO:*0051047).

3.1.3. Biological pathway analysis

Based on the KEGG database analyses, there were 33 biological pathways detected using the list of DE-genes. The results are compared in Table 2. The pathway with the largest number of genes was associated with *cancer* (eight DE-genes). The remaining pathways that were identified using the DAVID technique were the *PI3K-Akt signalling pathway* (seven DE-genes), *rheumatoid arthritis* (six DE-genes), *Rap1 signalling pathway* (five DE-genes) and *endocytosis* (five DE-genes). Less significant pathways included *insulin resistance*, *chemokine signalling pathway*, *Ras signalling pathway*, *cytokine-cytokine receptor interaction*, *MAPK signalling pathway* (four DE-genes), *glucagon signalling pathway*, *Toll-like receptor signalling pathway*, *AMPK signalling pathway*, *insulin signalling pathway*, *FoxO signalling pathway*, *Jak-STAT signalling pathway* (three DE-genes), and *fructose and mannose metabolism*, *mineral absorption*, *carbohydrate digestion and absorption* (two DE-genes).

Table 1
List of the most up- and down-regulated biological processes in the adiponectin-treated anterior pituitary cells of pigs, in comparison to the non-treated control group; GO: - unique gene ontology identification number; *Subset of the process; Bold font indicates the major processes; Complete gene names have been reported in SF1.

		Count	P-value	Altered genes
Up-regulated				
GO:0035556	**Intracellular signal transduction**	**16**	**5.03E-03**	**PIK3CG, RALGPS1, NPR2, PIK3IP1, CAPZB, TLR8, SLC11A1, MAP3K6, TEK, INPP5F, TPR, RASA3, RAPGEF2, DCX, GFRA2, F2R**
*GO:0000165	MAPK cascade	7	1.06E-02	PIK3CG, SLC11A1, MAP3K6, TEK, RAPGEF2, TPR, F2R
*GO:0048017	Inositol lipid-mediated signaling	5	4.62E-04	PIK3CG, TEK, INPP5F, PIK3IP1, F2R
*GO:0048015	Phosphatidylinositol-mediated signaling	5	4.18E-04	PIK3CG, TEK, INPP5F, PIK3IP1, F2R
*GO:0014065	Phosphatidylinositol 3-kinase signaling	4	2.54E-03	PIK3CG, TEK, PIK3IP1, F2R
GO:1902531	**Regulation of intracellular signal transduction**	**10**	**3.64E-02**	**PIK3CG, MAP3K6, RALGPS1, TEK, RAPGEF2, PIK3IP1, RASA3, CAPZB, GFRA2, F2R**
*GO:0014066	Regulation of phosphatidylinositol 3-kinase signaling	3	2.06E-02	TEK, PIK3IP1, F2R
GO:0051174	**Regulation of phosphorus metabolic process**	**9**	**9.66E-02**	**PIK3CG, PPARA, MAP3K6, TEK, RAPGEF2, PIK3IP1, TLR8, GFRA2, F2R**
*GO:0019220	Regulation of phosphate metabolic process	9	9.66E-02	PIK3CG, PPARA, MAP3K6, TEK, RAPGEF2, PIK3IP1, TLR8, GFRA2, F2R
*GO:0042325	Regulation of phosphorylation	9	4.90E-02	PIK3CG, PPARA, MAP3K6, TEK, RAPGEF2, PIK3IP1, TLR8, GFRA2, F2R
*GO:0043549	Regulation of kinase activity	6	4.67E-02	PIK3CG, MAP3K6, TEK, RAPGEF2, PIK3IP1, F2R
*GO:0033674	Positive regulation of kinase activity	5	2.88E-02	PIK3CG, MAP3K6, TEK, RAPGEF2, F2R
*GO:0045860	Positive regulation of protein kinase activity	4	8.95E-02	PIK3CG, MAP3K6, RAPGEF2, F2R
*GO:0043410	Positive regulation of MAPK cascade	5	3.63E-02	PIK3CG, MAP3K6, TEK, RAPGEF2, F2R
*GO:0070374	Positive regulation of ERK1 and ERK2 cascade	3	9.05E-02	TEK, RAPGEF2, F2R
*GO:0000186	Activation of MAPKK activity	2	7.29E-02	MAP3K6, F2R
GO:0044087	**Regulation of cellular component biogenesis**	**8**	**5.42E-03**	**PTGER4, CHMP5, TEK, MITF, RAPGEF2, TPR, CAPZB**
GO:0070271	**Protein complex biogenesis**	**7**	**8.44E-02**	**EPS15, PTGER4, TEK, MITF, SCARA5, CAPZB**
GO:0051338	**Regulation of transferase activity**	**7**	**2.27E-02**	**PIK3CG, MAP3K6, TEK, HMBOX1, RAPGEF2, PIK3IP1, F2R**
*GO:0051347	Positive regulation of transferase activity	6	1.12E-02	PIK3CG, MAP3K6, TEK, HMBOX1, RAPGEF2, F2R
GO:0005975	**Carbohydrate metabolic process**	**6**	**7.47E-02**	**PPARA, MAN2B2, SORD, GLB1L, PFKFB1, GYS2**
*GO:0005996	Monosaccharide metabolic process	4	1.50E-02	PPARA, MAN2B2, SORD, PFKFB1
*GO:0016051	Carbohydrate biosynthetic process	3	7.78E-02	PPARA, SORD, GYS2
GO:0001817	**Regulation of cytokine production**	**5**	**5.41E-02**	**PTGER4, ACP5, CLEC5A, TLR8, F2R**
*GO:0001819	Positive regulation of cytokine production	4	7.21E-02	PTGER4, CLEC5A, TLR8, F2R
*GO:0032651	Regulation of interleukin-1 beta production	2	9.46E-02	ACP5, F2R
*GO:0050707	Regulation of cytokine secretion	4	8.45E-03	PTGER4, CLEC5A, TLR8, F2R
*GO:0050715	Positive regulation of cytokine secretion	3	2.76E-02	PTGER4, CLEC5A, F2R
GO:0050708	**Regulation of protein secretion**	**4**	**6.56E-02**	**PTGER4, CLEC5A, TLR8, F2R**
*GO:0050714	Positive regulation of protein secretion	3	8.62E-02	PTGER4, CLEC5A, F2R
GO:0007168	**Receptor guanylyl cyclase signaling pathway**	**2**	**3.71E-02**	**NPR2, PDZD3**
GO:0043551	**Regulation of phosphatidylinositol 3-kinase activity**	**2**	**9.88E-02**	**TEK, PIK3IP1**
Down-regulated				
GO:0006955	**Immune response**	**8**	**3.61E-04**	**AMCF-II, IL6, TNFSF13B, FGB, FCN2, CD274, ANKHD1, IL1A**
*GO:0002684	Positive regulation of immune system process	4	5.43E-02	AMCF-II, FCN2, CD274, IL1A
GO:0006952	**Defense response**	**7**	**3.50E-03**	**AMCF-II, IL6, FGB, FCN2, ANKHD1, PTPRCAP, IL1A**
*GO:0002526	Acute inflammatory response	2	9.88E-02	IL6, IL1A
*GO:0006953	Acute-phase response	2	4.03E-02	IL6, IL1A
GO:0022603	**Regulation of anatomical structure morphogenesis**	**5**	**2.24E-02**	**WARS, NKX2-1, LPAR3, TBR1, IL1A**
*GO:0010769	Regulation of cell morphogenesis involved in differentiation	3	5.31E-02	NKX2-1, LPAR3, TBR1
GO:0040012	**Regulation of locomotion**	**4**	**5.19E-02**	**AMCF-II, NKX2-1, TBR1, IL1A**
*GO:0051270	Regulation of cellular component movement	4	5.73E-02	AMCF-II, NKX2-1, TBR1, IL1A
GO:0045165	**Cell fate commitment**	**3**	**3.52E-02**	**IL6, NKX2-1, TBR1**
*GO:0048663	Neuron fate commitment	2	7.73E-02	NKX2-1, TBR1
*GO:0021877	Forebrain neuron fate commitment	2	1.56E-02	NKX2-1, TBR1
GO:0051047	**Positive regulation of secretion**	**3**	**4.68E-02**	**IL6, CD274, IL1A**
GO:0061564	**Axon development**	**3**	**7.87E-02**	**NKX2-1, LPAR3, TBR1**
*GO:0007409	Axonogenesis	3	8.50E-02	NKX2-1, LPAR3, TBR1
GO:0032642	**Regulation of chemokine production**	**2**	**6.45E-02**	**AMCF-II, IL1A**
GO:0021872	**Forebrain generation of neurons**	**2**	**7.00E-02**	**KX2-1, TBR1**
*GO:0021879	Forebrain neuron differentiation	2	6.08E-02	**NKX2-1, TBR1**

*-Subset; Bold font indicate the major process;

Table 2
KEGG pathway enrichment of pituitary genes of pigs affected by treatment with adiponectin; Complete gene names have been reported in SF1.

KEGG pathway analysis name	Gene number	*P*-value	Altered genes
Pathways in cancer	8	6.2E-3	PIK3CG, IL6, PTGER4, SOS1, MITF, LPAR3, TPR, F2R
PI3K-Akt signaling pathway	7	8.6E-3	PIK3CG, IL6, SOS1, TEK, GYS2, LPAR3, F2R
Rheumatoid arthritis	6	3.3E-5	AMCF-II, IL6, TNFSF13B, TEK, ACP5, IL1A
Rap1 signaling pathway	5	1.6E-2	PIK3CG, TEK, LPAR3, RAPGEF2, F2R
Endocytosis	5	3.5E-2	EPS15, CHMP5, CAPZB, F2R
Insulin resistance	4	8.6E-3	PIK3CG, PPARA, IL6, GYS2
Osteoclast differentiation	4	1.5E-2	PIK3CG, MITF, ACP5, IL1A
Non-alcoholic fatty liver disease (NAFLD)	4	2.8E-2	PIK3CG, PPARA, IL6, IL1A
Chemokine signaling pathway	4	3.6E-2	AMCF-II, PIK3CG, CCR6, SOS1
Ras signaling pathway	4	7.4E-2	PIK3CG, SOS1, TEK, RASA3
Cytokine-cytokine receptor interaction	4	7.5E-2	IL6, CCR6, TNFSF13B, IL1A
MAPK signaling pathway	4	1.0E-1	MAP3K6, SOS1, RAPGEF2, IL1A
Pertussis	3	1.6E-2	AMCF-II, IL6, IL1A
Glucagon signaling pathway	3	3.1E-2	PPARA, PFKFB1, GYS2
HIF-1 signaling pathway	3	3.6E-2	PIK3CG, IL6, TEK
Toll-like receptor signaling pathway	3	3.6E-2	PIK3CG, IL6, TLR8
AMPK signaling pathway	3	5.3E-2	PIK3CG, PFKFB1, GYS2
Hepatitis C	3	6.4E-2	PIK3CG, PPARA, SOS1
Platelet activation	3	6.8E-2	PIK3CG, FGB, F2R
Insulin signaling pathway	3	6.9E-2	PIK3CG, SOS1, GYS2
Measles	3	7.0E-2	PIK3CG, IL6, IL1A
FoxO signaling pathway	3	7.2E-2	PIK3CG, IL6, SOS1
Jak-STAT signaling pathway	3	8.1E-2	PIK3CG, IL6, SOS1
Dorso-ventral axis formation	2	1.6E-2	LOC100520559, SOS1
Fructose and mannose metabolism	2	2.0E-2	SORD, PFKFB1
Prion diseases	2	2.4E-2	IL6, IL1A
Graft-versus-host disease	2	2.5E-2	IL6, IL1A
Mineral absorption	2	3.7E-2	SLC11A1, SLC5A1
Carbohydrate digestion and absorption	2	3.7E-2	PIK3CG, SLC5A1
Intestinal immune network for IgA production	2	3.9E-2	IL6, TNFSF13B
Notch signaling pathway	2	4.0E-2	LOC100520559, PTCRA
Endometrial cancer	2	4.9E-2	PIK3CG, SOS1

3.1.4. Network of interactions between differentially expressed genes

An results from the analysis of the interactions between the genes affecting the synthesis, metabolism and function of hormones and other biologically active substances indicated that all genes were connected within a single network (Fig. 1). There was gene co-expression in 45 interactions, and co-localisation in seven interactions. In 18 cases, selected genes contributed to modulation of common pathways. There were physical interactions in 42 cases; and in 11 cases, the interactions were based on shared protein domains. Genetic interactions between selected genes were not noted. A complete list of interactions is presented as supplementary data (Supplementary file SF.5.).

3.2. Real-time PCR validations

Microarray data were validated by selecting nine genes with FC above 1.2 for qPCR. The products of the selected genes had important functions at the anterior pituitary functions such as intracellular signal transduction (DPYS, GABBR2), metabolism of lipids, carbohydrates and proteins (SLC5A1, WARS), prostaglandin synthesis and metabolism (PTGER4), interleukin and growth factor activity (PPARA, GRB14, TNFSF13B), and natriuretic peptide actions (NPR2). The DNA microarray data were validated using qPCR to confirm changes in the expression of all examined genes. The changes in gene expression determined by the DNA microarray were confirmed by qPCR (Fig. 2).

4. Discussion

The primary aim of this study was to determine the effect of adiponectin on global gene expression in anterior pituitary cells of pigs and on differentially expressed genes (DE-genes). Results from previous studies indicated adiponectin had an effect on pituitary secretory functions; however, these studies were conducted on a predetermined group of genes and gene products, and there was no examination of the entire transcriptome. In a study by Kiezun et al. (2014), adiponectin modulated basal FSH secretion *in vitro* during the oestrous cycle. Adiponectin did not, however, affect basal LH secretion of AP cells of pigs. Furthermore, adiponectin's effect on GnRH- and/or insulin-induced secretion of gonadotrophins *in vitro* was dependent on the stage of the oestrous cycle and the adipocytokine dose. The secretory functions of AP cells of pigs could also be affected by the relatively long time (24 h) of incubation with this adipocytokine. Results of a study of the AP cells of male rats indicated that short-term (4 h) treatment with adiponectin decreased basal and GnRH-stimulated LH secretion. In contrast, long-term treatment with the adipocytokine did not affect the capacity of

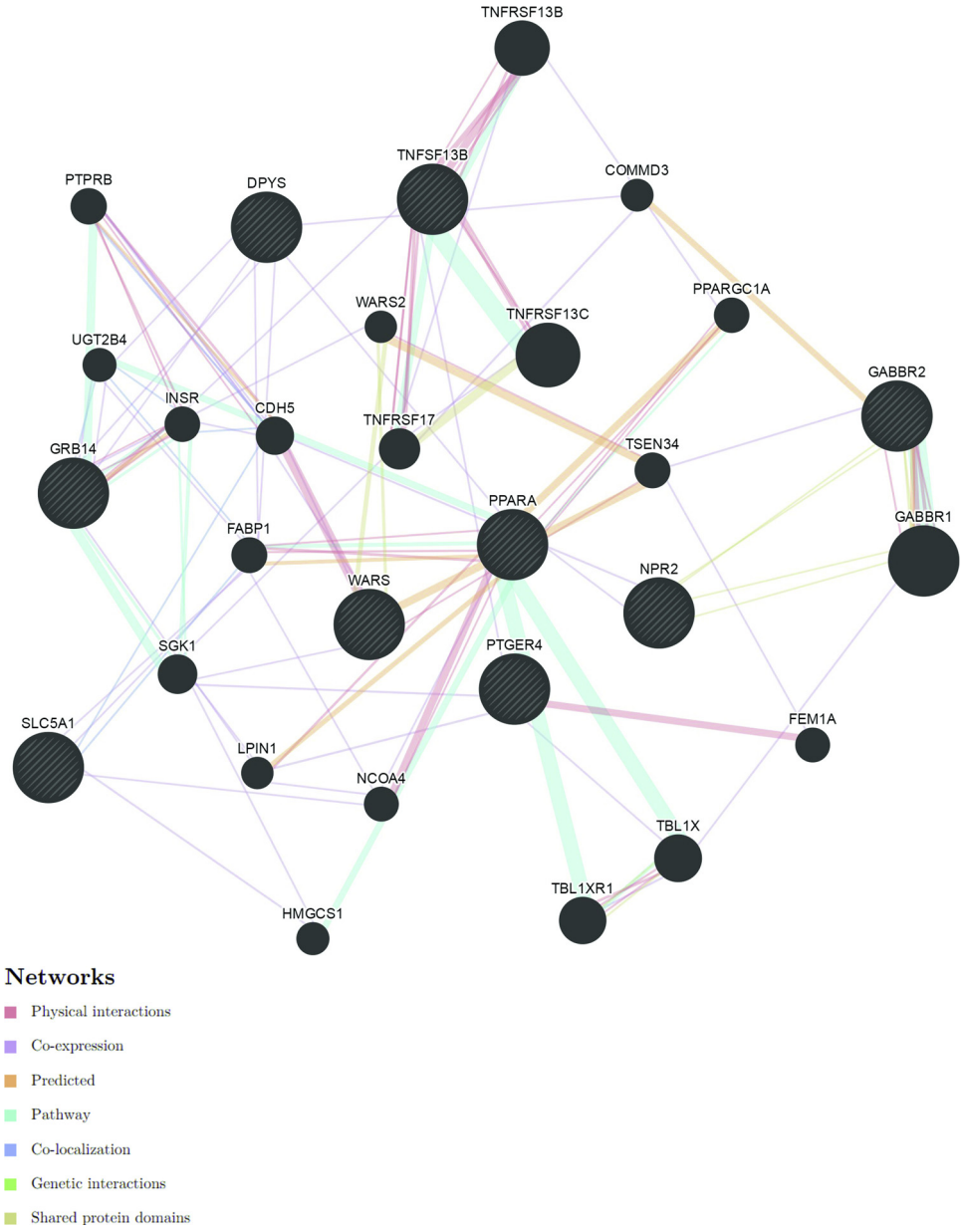

Fig. 1. Network of interactions between selected genes as assessed using GeneMania; Network consists of 29 total genes (circles) and 123 total interactions (lines); Input genes are indicated with stripes; Different lines and colours denote the different type of interactions: in purple co-expression, in orange predicted, in blue co-localisation, in green genetic interactions, in light red physical interactions, in light blue pathway and in light green shared protein domains.

GnRH-stimulated AP cells to release LH, regardless of the hormone dose (Rodriguez-Pacheco et al., 2007). These findings imply that adiponectin's effect on AP cells is species-specific and time-dependent. Even though there are species- and sex-specific differences, these observations are consistent with the results in pigs, and indicate adiponectin has an important role in the auto/paracrine regulation of AP functions, including gonadotrophin secretion.

Adiponectin receptors (AdipoRs) activate adenosine monophosphate-activated protein kinase (AMPK), peroxisome proliferator-activated receptor alpha (PPARα), mitogen-activated protein kinase (MAPK) (Yamauchi et al., 2003) and phosphatidylinositol 3-kinase (PI3K) pathways (Su et al., 2016). The expression of AdipoRs genes was confirmed in the AP of gilts (Kiezun et al., 2013) and female rats (Kim et al., 2013), and it was also noted in the somatotroph cells of mice and LβT2 cells (immortalised mouse gonadotrophs) (Lu et al., 2008). The AMPK protein has functions in restoring cellular ATP concentrations by inhibiting anabolic pathways, stimulating glucose uptake and fatty acid β-oxidation (Towler and Hardie, 2007). Little is known, however, about adiponectin's AMPK-mediated effects on reproduction. Lu et al. (2008) hypothesised that adiponectin affects the reproductive system by

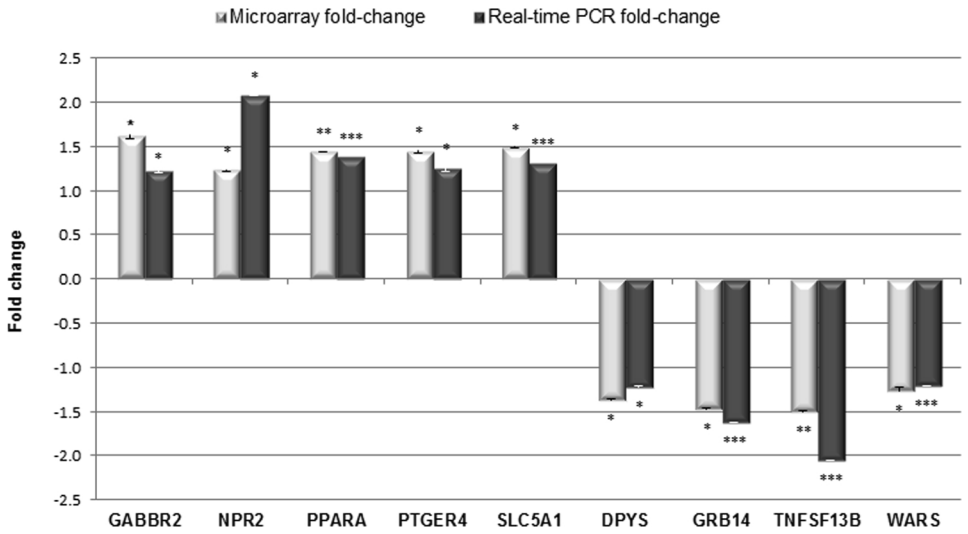

Fig. 2. qPCR validation of the microarray experiment; Light grey bars represent fold changes for microarray data; dark grey bars represent fold changes for qPCR data; Data are presented as means ± SEM of fold changes from four different observations; Differences in gene expression between non-treated (control) and adiponectin-treated (10 μg/ml) anterior pituitary cells measured by the qPCR method were analysed using one-way ANOVA, followed by use of the least significant difference (LSD) *post-hoc* test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

modulating gonadotroph functions. Results of this previous study indicated that in LβT2 pituitary gonadotroph cells there was expression of adiponectin receptors that responded to adiponectin by phosphorylating AMPK. The results from this study could imply that pituitary AMPK functions as a biological sensor which is involved in sensing ATP concentrations in cells, the nutritional status of the body and, indirectly, the secretion of gonadotrophins to control reproduction processes. In the present study, there was identification of three DE-genes associated with the AMPK signalling pathway which were up-regulated by adiponectin: phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit gamma (*PIK3CG*), 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1 (*PFKFB1*) and glycogen synthase 2 (*GYS2*). The PIK3CG gene product phosphorylates inositol lipids and affects the immune response (Kächele et al., 2015). The PFKFB1 catalyses both the synthesis and degradation of fructose-2,6-biphosphate, which activates the glycolysis pathway and inhibits the gluconeogenesis pathway. By regulating fructose-2,6-biphosphate concentrations, the enzyme regulates carbohydrate metabolism in cells (Batra et al., 1997). The *GYS2* gene encodes liver glycogen synthase and catalyses the rate-limiting step in glycogen synthesis whereby the glucosyl moiety is transferred from UDP-glucose to a terminal branch of the glycogen molecule (Weinstein et al., 2006). The above suggests that adiponectin could exert an indirect effect on the AMPK signalling pathway by modulating the expression of these three genes.

Adiponectin also affects the following groups of genes which encode intracellular signal transduction in AP cells of pigs: *MAPK cascade (GO:*0000165*)* and *phosphatidylinositol 3-kinase signalling (GO:*0014065*)*. The first group of seven genes is associated with intracellular signal transduction. Mitogen-activated protein kinase (MAPK) is an element of one of the major pathways which transduce signals from the cell surface to the nucleus, and control cellular responses. The evidence for the important role of MAPK ERK signalling in gonadotroph functions was provided mainly in a study of αT3–1 and LβT2 gonadotroph-derived cell lines. To the best of our knowledge adiponectin's effects on reproductive functions that are modulated by the MAPK ERK pathway are limited to the hypothalamus and the ovaries. In an *in vitro* study, adiponectin decreased hypothalamic GnRH secretion by activating the AMPK pathway and inhibiting the ERK pathway (Cheng et al., 2011). The activation of the MAPK ERK1/2 pathway is a necessary step for the induction of steroidogenesis and steroidogenic gene expression in granulosa cells (Moore et al., 2001; Dewi et al., 2002).

Adiponectin also up-regulated a group of genes that encode the *regulation of phosphatidylinositol 3-kinase signalling (GO:*0014066*)*. In the present study, three genes that belong to this group were identified: *TEK, F2R* and *PIK3IP1*. Phosphatidylinositol 3-kinases (PI3K(s)) are a family of enzymes that modulate cellular functions such as cell growth, proliferation, differentiation, motility, survival, intracellular signalling and, consequently, carcinogenesis (Vanhaesebroeck et al., 2012). The first gene, *TEK* encodes the angiopoietin-1 receptor which functions to regulate angiogenesis, endothelial cell survival, actin cytoskeleton organisation, and maintenance of vascular quiescence (Chavakis and Dimmeler, 2002). It seems that adiponectin can indirectly induce angiogenesis in the anterior pituitary by regulating *TEK* gene expression. The second gene, coagulation factor II receptor (*F2R*), is a G protein-coupled receptor which functions in the regulation of thrombotic response. The third gene, *PIK3IP1*, encodes PI3K signalling pathways in AP cells. An increase in *PIK3IP1* gene expression as a result of the effects of adiponectin, could lead to inhibition of the PI3K signalling pathway.

In the current study, treatment with adiponectin also increased the expression of *PPARA* and peroxisome proliferator-activated receptor gamma coactivator 1-alpha *(PPARGC-1)* genes. The PPAR family of proteins function in the regulation of energy homeostasis, in particular fatty acid oxidation and carbohydrate metabolism (Contreras et al., 2013), and have an important role in reproduction. In the studies of *PPARA*-null mice PPARα increased pituitary expression of prolactin (*PRL*) and *LHβ* genes during

fasting (König et al., 2009). It cannot be discounted that by affecting *PPARA* gene expression, adiponectin indirectly regulates the abundance of *PRL* and *LHβ* mRNA.

The results of the gene ontology analysis in the present study indicate there are adiponectin-regulated genes that could modulate the production and secretion of biologically active molecules by AP cells of pigs. Three DE-genes were identified in the present study: prostaglandin E receptor 4 (*PTGER4*), C-type lectin domain containing 5A (*CLEC5A*) and F2R (already mentioned), which are associated with the *positive regulation of protein secretion (GO:*0050714*)*. The *PTGER4* gene product is a receptor for prostaglandin E_2 (PGE_2). The expression of the *PTGER4* gene in AP cells could be a consequence of paracrine mechanisms of prostaglandin E action on the secretory functions of the pituitary gland. The *CLEC5A* is yet another gene which is up-regulated by adiponectin and which could affect the regulation of protein secretion in AP cells of pigs. A product of the *CLEC5A* gene is a receptor that participates in the regulation of inflammatory responses and has an important role in the activation of macrophages (Watson et al., 2011).

The group of genes that were down-regulated by adiponectin is associated with *positive regulation of secretion (GO:*0051047*)*. This group consists of a cluster of differentiation 274 (*CD274*), interleukin 6 (*IL6*) and interleukin 1 alpha (*IL1A*) genes. The CD274 gene product is an immune inhibitory receptor ligand for which the gene is expressed by hematopoietic and non-hematopoietic cells, such as T and B lymphocytes and several types of tumour cells. Recent evidence indicates that adiponectin functions mainly as an anti-inflammatory reactant. Adiponectin induces the secretion of anti-inflammatory cytokines, such as IL10 and IL1RA (receptor antagonist), by human monocytes, macrophages and dendritic cells and suppresses the secretion of the pro-inflammatory IL6, tumour necrosis factor alpha (TNFα) and IFNγ (Wolf et al., 2004; Wulster-Radcliffe et al., 2004). The results of the present study also indicate that adiponectin inhibits the expression of the *IL6* gene in the AP of pigs. The IL6 protein is a multifunctional cytokine that has an important role in the regulation of cell growth, and is also capable of inducing the secretion of GH, PRL, adrenocorticotrophin (ACTH) and LH/FSH in the rat pituitary gland *in vivo* and/or *in vitro* (Fukata et al., 1989; Spangelo et al., 1989). Another gene down-regulated by adiponectin is *IL1A* which is associated with *positive regulation of secretion (GO:*0051047*)*. In *in vitro* studies of cells from the pituitary gland of sheep, IL1A stimulated LH release (Braden et al., 1998). The results of the present study provide evidence that adiponectin could affect the production and secretion of biologically active molecules by AP cells of pigs by modifying the expression of *PTGER4, CLEC5A, F2R*, *CD274*, *IL6* and *IL1A* genes.

5. Conclusion

This study was conducted to evaluate adiponectin's effect on global gene expression in the anterior pituitary cells of pigs during the mid-luteal phase of the oestrous cycle. This is the first study where there was identification of a group of genes for which expression is affected by adiponectin and which is potentially implicated in signal transduction, regulation of metabolism and female reproductive functions. The presented results expand the knowledge base of the role of adiponectin in modulating the secretion of reproductive hormones from the anterior pituitary of pigs. Nevertheless, further functional studies are needed to enhance the understanding of the role and mechanism of adiponectin action in AP cells of pigs.

Declarations 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: GSE122311).

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.2019.05.002.

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