POLYMORPHISM OF *OPN* AND *AREG* GENES IN RELATION TO TRANSCRIPT EXPRESSION OF A PANEL OF 12 GENES CONTROLLING REPRODUCTION PROCESSES AND LITTER SIZE IN PIGS*

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Abstract

The aim of this study was to define the transcript expression of 12 genes, identify new polymorphisms in selected 2 genes and to estimate the association between the level of expression, gene polymorphism and litter size in sows. Two groups of sows were selected: 71 crossbred sows and 328 purebred sows. For transcript analysis endometrial tissue samples were collected, while blood was sampled for the purpose of DNA polymorphism analysis. For all animals data on litter size and weaned piglets were obtained. Transcript analysis of 12 genes was performed in the uterine endometrium of sows in the luteal and follicular phases. Eight out of 12 genes showed higher mRNA expression levels during the luteal phase (*AREG*, *FABP3*, *IL1A*, *ITGAV*, *ITGB3*, *NMB*, *OPN*, *RBP4*). In turn, higher expression levels in the follicular phase were observed for 4 genes (*IL1B*, *PPARG*, *SI00A8*, *SELL*). Analysis of six new polymorphic sites within the *OPN* and *AREG* genes revealed the highest heterozygosity for OPNe6_Knoll, OPNp3_617 and AREGe2_317 polymorphisms and the lowest heterozygosity in the AREGe3_561 locus. Association analyses concerning transcript expression levels of the 12 genes and the *OPN* and *AREG* genotypes in the two groups of sows showed a significant relationship between the *IL1A*, *ITGB3* transcript expression and the OPNe7_603 genotype also between OPNp3_617 genotype and *ITGB3* transcript expression. With regard to the litter size and the number of weaned piglets a significant relation with the OPNp3_617, OPNe6_462 and AREGe2_317 genotypes was observed only in the purebred sows. Transcript expression of the genes encoding osteopontin and amphiregulin in the uterus of the sows affect reproductive traits by preparing the uterus for embryo reception.

Key words: pigs, gene polymorphism, gene expression, *OPN*, *AREG*, reproduction

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From an economic point of view, reproductive efficiency is one of the most important factors in livestock production. The reproductive performance of a sow is one of the key factors affecting pig production profitability (Onteru et al., 2011). Unlike other livestock species, the pig is a multiparous species that gives birth to a large number of offspring at the same time. In prolific species, such as pigs, an important trait is related with litter size, especially the number of piglets born alive (NBA). Genetic improvement of reproductive traits in sows is difficult and complex due to a low heritability and sex-limited expression (Zak et al., 2017). Fertility traits are also affected by season, parity and breed, as well as housing and feeding conditions (Knecht et al., 2015). Litter size is a complex physiological trait, influenced by several components that are expressed sequentially, i.e. ovulation, fertilization, embryo development and fetal survival (Argente, 2016). However, little is still known about the underlying genetics and the molecular basis of each step involving these general processes.

Several studies have investigated the biological basis of NBA in pig production. The analysis of individual genes, even with a significant documented effect of their polymorphism on the trait, does not provide a full picture of the regulation of a given process. The latest methods of molecular genetics enable a comprehensive analysis of the entire genome, both at the structural (for example SNP – Single Nucleotide Polymorphism) and functional (gene expression-mRNA) levels. Therefore, the analysis of mRNA expression profiles allows us to determine causes for changes in tissues at the molecular level. Analysis of differences in the expression of these genes during the oestrous cycle can be used to select genes and pathways important for uterine receptivity (Bauersachs and Wolf, 2012). Currently, both genomic and transcriptomic (microarray and RNA sequencing) approaches have been used to analyse quantitative trait loci (QTL) and to assess candidate genes for reproductive traits in pigs (Kumchoo and Mekchay, 2015). Several genes have been discovered: *FSHB*, *ESR*, *PRLR*, *GDF9* and *MTNR1A* (Zhang et al., 2015). Based on our previous research and microarray data a total of 12 genes were selected for further analyses (Pierzchała et al., 2020). All these genes are involved in the regulation of common biological processes (immune response, angiogenesis, lipid metabolism, cell adhesion, intercellular transmission and response to stimuli) as well as regulation at the level of expression (coexpression) and subject to common regulation within the genome (co-localisation analysis) (Pierzchała et al., 2020). The mRNA expression level was analysed for *AREG* (amphiregulin), *FABP3* (fatty acid-binding protein 3), *IL1A* (interleukin 1A), *IL1B* (interleukin 1B), *ITGAV* (av integrin), *ITGB3* (β3 integrin), *NMB* (neuromedin B), *OPN* (osteopontin), *PPARG* (peroxisome proliferator-activated gamma receptor), *RBP4* (retinol-binding protein 4), *SI00A8* (calgranulin A) and *SELL* (selectin). From this group of genes, two were selected for polymorphism analyses. One is osteopontin (*OPN*), which can be differentially expressed in various reproductive tissues, e.g. ovarian follicles, ovaries, myometrium and endometrium (Fernandez-Rodriguez et al., 2011; Sun et al., 2011; Wang et al., 2013; Samborski et al., 2013). The *OPN* (named osteopontin or secreted phosphoprotein 1-*SPPI*) is an extracellular matrix (ECM) protein and integrin-binding ligand, as well as a highly phosphorylated acidic glycoprotein that stimulates cell-cell adhesion, increases cell–

ECM communication and promotes cell migration, cell signaling and remodeling in the uterus and placenta (Garlow et al., 2002; Johnson et al., 2003; White et al., 2005). The other investigated gene was *AREG* (amphiregulin), which belongs to the family of epidermal growth factors and is a growth modulator (Shoyab et al., 1989). The addition of amphiregulin to the culture medium results in an increased proliferation of porcine embryo trophoblast cells *in vitro* during the preimplantation period (Lee et al., 2009). *AREG* is regulated by LH (luteinizing hormone) (Zamah et al., 2010) and its expression is influenced by P4 (Progesterone), but only at the place of implantation (Das et al., 1995). *AREG* also stimulates the maturation of porcine oocytes and the expansion of *cumulus* cells *in vitro*; however, while in that way it is similar to FSH, it is weaker. The *AREG* expression is also stimulated by FSH (follicle-stimulating hormone) (Procházka et al., 2011). Amphiregulin overexpression, under the influence of LH and to a lesser extent FSH, seems necessary for ovulation and luteinisation in the human ovary. It was observed that amphiregulin expression in ovarian granular cells positively correlated with LH and FHS induced progesterone levels (Freimanna et al., 2004).

In light of what is known about the processes occurring in the reproductive system of sows and the previous research of this group of authors (Pierzchała et al., 2020) a research hypothesis was adopted, which assumes that genes, whose expression is regulated around implantation may affect litter size and piglet survival. Thus, such genes can become markers of reproductive characteristics in sows.

The aim of this study was to identify polymorphisms in selected pig's genes and estimate their association with the level of gene expression, as well as determine their relationship with reproductive traits.

Material and methods

Animals

Two groups of pigs were selected for the purpose of:

- 1) polymorphism and transcript analyses – the first group consisted of 71 Polish Large White (PLW) × Polish Landrace (PL) F1 designated crossbred sows. Animals were kept under identical environmental conditions and fed a uniform diet. The sows were slaughtered 10 days after weaning of their first litter. At the time of slaughter the animals were divided into two sub-groups based on the phase of the oestrous cycle (actually, based on the observation of morphological changes in the ovary and the *corpus luteum*). Shortly after slaughter both ovaries from each sow were photographed in four different projections, which were used to assess the cycle phase of the sow at the time of slaughter: group L (luteal phase, n = 46) or group F (follicular phase, n = 25). For gene expression analysis by the Real-time PCR method endometrial specimens were collected from the central mesometric side of the uterine horn, frozen in liquid nitrogen and stored at –80°C until analysis. For DNA polymorphism

analysis blood samples in the amount of 2 ml were collected into tubes containing EDTA anticoagulant and stored at -20°C until analysis.

2) genotyping – the other group of animals consisted of 328 Polish Large White (PLW) purebred sows, collected from four farms in the Kuyavian-Pomeranian Province and made available by the University of Science and Technology in Bydgoszcz, the Faculty of Animal Breeding and Biology. Blood samples for DNA genotyping were collected into tubes containing EDTA anticoagulant and stored at -20°C until the time of further analysis

For all animals from both groups the following data on reproductive performance were collected: the number of piglets born alive (NBA) in the litter and the number of piglets weaned on day 21 (NPW). The data concerned the first litter. All veterinary interventions were conducted by a veterinary surgeon according to the veterinary procedures specified by the National Ethical Committee for Animal Experiments (Decision No. 13/2010 of the 3rd Local Ethics Committee).

Molecular methods

Isolation of nucleic acids

RNA

After slaughter the reproductive system was dissected. The tissue sections were prepared from a similar location in the horn of the uterus. Afterwards RNA was isolated from samples containing approximately 50 mg of uterine endometrium. Samples were homogenised on the MagNALyser (Roche Diagnostics Corporation) in Qiazol buffer (Qiagen, Valencia, CA, USA) in three cycles. RNA was isolated from tissues using the RNeasy Microarray Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The isolates were then digested with RNase-free DNase (Sigma-Aldrich Co. LLC) in accordance with the manufacturer's protocol to remove the remaining genomic DNA. Isolated RNA samples were stored at -80°C . Initial quantitative and qualitative RNA analyses were performed based on spectrophotometric measurements (NanoDrop ND-1000 apparatus from ThermoScientific, Pittsburgh, PA, USA) and electrophoresis (in 1% denaturing agarose gel with the addition of ethidium bromide). Finally, the integrity of the isolated RNA was verified using a BioAnalyzer 2100 instrument and the Agilent 2100 Expert software (Agilent Technologies, Santa Clara, USA). For further analysis samples above 8 RIN (RNA integrity number) and absorbance in the range of 1.8–2.0 were selected.

DNA

DNA was isolated from 200 μl of peripheral blood using a chloroform method and cleaned on Qiagen (DNeasy) columns following the manufacturer's protocol. The quantitative and qualitative evaluation of isolated nucleic acids was carried out using the NanoDrop 1000 apparatus (Thermo Scientific, Pittsburgh, PA, USA). Isolated DNA samples were stored at -20°C .

Transcript expression analysis (real-time PCR)

The transcript expression levels of 12 selected genes were analysed in the endometrium of the uterine horns of sows from L and F groups (Table 1). The analy-

sis was performed by the standard real-time PCR method with the double-stranded DNA dye, EvaGreen. For this purpose the Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) was used to enable simultaneous verification of primer specificity. The quality of the primers was verified using the OligoAnalyzer 3.1 software (Integrated DNA Technologies, US – <https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>). The reaction primers were designed to span the same region as the probe in the previous microarray analysis.

Table 1. Primer sequences and GenBank accession numbers of selected genes

Gene	Primer sequences	GenBank accession number
<i>AREG</i>	F: 5' - AGGTGCAGCTGAAGAACGAA - 3' R: 5' - TATGATCCACTGCAAAGAGGAA - 3'	NM_214376
<i>FABP3</i>	F: 5' - GGCCAACATGACCAAGCCTA - 3' R: 5' - CTGCCATGGGTGAGTGTCTAG - 3'	NM_001099931
<i>RBP4</i>	F: 5' - CCCCAGGGACTCTTTCTGC - 3' R: 5' - TCTTGAACCTGGCAGGGTCC - 3'	NM_214057
<i>PPARG</i>	F: 5' - TTAGATGACAGCGACCTGGC - 3' R: 5' - GTGAAGGCTCATGTCCGTCT - 3'	NM_214379
<i>IL1A</i>	F: 5' - AACCTGGATGAGGCAGTGAAAT - 3' R: 5' - AACACGGGTTCTGCTTCGTT - 3'	NM_214029
<i>IL1B</i>	F: 5' - GTACATGGTTGCTGCCTGAA - 3' R: 5' - TGGCACACTCACCCCAAAG - 3'	NM_214055
<i>OPN</i>	F: 5' - CACATTGTAGCGAGGTGGGA - 3' R: 5' - GTGACGGCTTGATTTCTTATACGG - 3'	NM_214023
<i>ITGB3</i>	F: 5' - GAAGAGTTTCTCCGTCCAAG - 3' R: 5' - TCATAGCAGGGGTTTTTGAG - 3'	NM_214002
<i>ITGAV</i>	F: 5' - GACTTCACTGCTGATGGTGC - 3' R: 5' - ACAAAAGATGTGGATAGTAAGGTCT - 3'	NM_001083932
<i>SELL</i>	F: 5' - AGCAAAGACTCCGGGAAGTG - 3' R: 5' - CCCGTAGTACCCTGCATCAC - 3'	NM_001112678
<i>NMB</i>	F: 5' - TGTGATTCTGGTTGAGTTGCC - 3' R: 5' - CAAGACATACAGCAGGGACG - 3'	NM_001123145
<i>S100A8</i>	F: 5' - AAGAGCTGGACATCAACAAGGA - 3' R: 5' - GCCCCAAAGCTCTGCTACTC - 3'	NM_001160271

F – forward primer, R – reverse primer.

The isolated total RNA was transcribed into complementary cDNA using the Transcription First Strand cDNA Synthesis Kit (Roche Life Science) in accordance with the manufacturer's procedure. The reaction mixture in a volume of 15 µl con-

tained 140 nM of each primer. Expression analysis was performed on a Light-Cycler 480 instrument (Roche Life Science) using 5xHOT FIREPol EvaGreen qPCR Mix Plus (no ROX) (Solis BioDyne, Estonia). The reaction conditions were optimised separately for each pair of primers. Amplification was carried out in 42 cycles: 95°C for 15 s, 54°C for 20 s and 72°C for 20 s, preceded by preliminary denaturation at 95°C for 15 minutes. The next step consisted in the analysis of melting curves for each product. The reaction was controlled using NTC (non-template control) and electrophoresis of reaction products in a 2% agarose gel. Two replicates were analysed for each sample.

Analysis of stability of the reference genes

Genes of basic metabolism, used as reference genes in the gene expression analysis by real-time PCR, may be influenced by steroid hormones, hence the need to verify the stability of expression for selected genes in the endometrium. Stability of nine reference genes (TBP, GAPDH, HPRT1, BACT, CYTB, YWHAZ, B2M, SDHA, HMBSA) was tested using the GenEX 5.4.4 software (MultiD Analyses AB) of the GeNorm (Ye et al., 2012) and NormFinder applications (OligoAnalyzer: Users Guide and Protocols, 2019). In the case of GeNorm the levels of expression of the reference genes were compared in pairs concerning the expression of other genes. The variability in the expression of a single gene was visualised using the value of M, which is the average standard deviation for each of the gene pairs. In the case of Norm Finder, the stability of gene expression was determined by the standard deviation (SD) of each gene. After evaluation check and stability measurements of the nine reference genes, both GeNorm and Norm Finder identified the TBP gene as the reference gene with the highest expression stability, while the GAPDH reference gene ranked second. However, the other seven reference genes were found to be the less stable. Therefore, a panel of both TBP and GAPDH was finally selected as the reference genes for further validation analysis using RT-PCR (Pierzchała et al., 2020).

Gene polymorphism examination – high resolution melting

The High Resolution Melting Analysis method was selected for the analysis of gene polymorphism in view of its speed and accuracy. The polymorphism analysis was performed for two genes *OPN* – promotor and coding sequence, also *AREG* – coding sequence. The analyses were based on the sequences available in GenBank. For *OPN Sus scrofa* 5' end (M 84121.1), partial exon 6 and exon 7 (AJ237667.1) and for *AREG – Sus scrofa* mRNA (NM_214376) and *AREG* human mRNA (NM_001657). For each of the genes the primers were designed using the Primer-BLAST software (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) enabling simultaneous verification of primer specificity. The quality of the primers was tested using the OligoAnalyzer 3.1 (Integrated DNA Technologies, US – <https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/>). The existence of polymorphism was established based on a scan of a population of crossbred sows. Next, when the polymorphic sites were identified, 328 individuals of the purebred group were genotyped (Table 2).

Table 2. Sequences of selected primers and the position of identified polymorphic sites

Gene	Mutation site	Fragments	Primer sequence
<i>OPN</i>	promoter	OPNp1-2	F: 5' - TGAGAGCCAATCCTATTACAG - 3' R: 5' - TAAAGGCTGCACCAGACATC - 3'
	promoter	OPNp3-4	F: 5' - AGTAACCTGCCCTCAGTCA - 3' R: 5' - TGCCAGCTTCTCTCCATTTC - 3'
	exon 1	OPNe1-1	F: 5' - TTAAATTCAAGGGGAGGTCCA - 3' R: 5' - CTACGACAGCAACCATCAGC - 3'
	exon 6	OPNe6-1	F: 5' - ACGGGAGACCCCAATGAT - 3' R: 5' - GCTGCGTCTGTTTCAGGATT - 3'
	exon 7	OPNe7-1	F: 5' - TGGATGCCACAGAGGAAGAC - 3' R: 5' - GGCTGACTCGTCTCCTGACT - 3'
	exon 7	OPNe7-2	F: 5' - CATCCCTGGCTGTTCATTTA - 3' R: 5' - CATCCGTCTCCTCACTTTCC - 3'
<i>AREG</i>	exon 1	ARE1-1	F: 5' - CCTATAAAGCCACTGGTGCG - 3' R: 5' - GAGGATCAAGAGTGACAGCACC - 3'
	exon 2	ARE2-1	F: 5' - CATTATGCTGCTGGACTG - 3' R: 5' - TCACCTTACCCTCTACTCAC - 3'
	exon 3	ARE3-1	F: 5' - AATACTTCAGATAAACCCAA - 3' R: 5' - GTTACTGCTTCCAGGTGC - 3'
	exon 4	ARE4-1	F: 5' - TTCAGATGTTACCAGGAT - 3' R: 5' - GAGACAAAAGCAGCAATG - 3'
	exon 5	ARE5-1	F: 5' - ACGATACTTCAGGGAATA - 3' R: 5' - AAAACTCAAACCTGTAAT - 3'
	exon 6	ARE6-1	F: 5' - ACTGCCAAGTCATAACCA - 3' R: 5' - TACCCTCAAGTCTCACAATC - 3'

F – forward primer, R – reverse primer.

Statistical analysis

Raw data concerning gene transcript expression was converted in the LC480 Conversion program (HFRC, The Netherlands). Next, the reaction efficiency was determined individually for each sample and each amplicon, using the LinRegPCR 12.10 application (HFRC, The Netherlands) (Ruijter et al., 2009). The relative level of expression was determined based on the differences in $C_p(\Delta C_p)$ with the correction of the amplification efficiency (E), using the following mathematical model:

$$Ratio = (E_{target})^{-\Delta C_p \text{ target (control-sample)}} / (E_{ref})^{-\Delta C_p \text{ ref (control-sample)}}$$

The results of the expression analysis were normalised based on two reference genes. The significance of differences in the relative level of gene expression between the analysed groups was determined based on the t-test for heterogeneous variances, the so-called Cochran and Cox tests (Cochran and Cox, 1957).

Pearson's analysis was performed between the transcript expression value of the studied genes and the reproductive traits related to the litter size, i.e. NBA and NPW. The analysis was performed in relation to the expression value depending on the cycle phase (luteal and follicular) in the crossbred gilts.

For identified gene polymorphisms the allele frequency, heterozygosity (H) and the polymorphic information content (PIC) were estimated. The analysis of the consistency of genotype distribution in the population with that expected from the Hardy–Weinberg law was performed. Next, using the GLM procedure of the SAS/STAT package (SAS Institute Inc., Cary, USA) (F statistic), a multivariate analysis of the variance was conducted, according to a model taking into account the effect of all the polymorphisms identified in the analysed genes on gene expression and reproductive traits.

The statistical model of analysis was :

$$Y_{ijk} = \mu + G_i + AG_j + e_{ijk}$$

where:

Y_{ijk} = analysed trait,

μ = overall mean,

G_i = fixed effect of the i -th genotype,

AG_j = fixed effect of the j -th animal group,

e_{ijk} = random error.

Variables before analysis were transformed into natural logarithm (LN(x)). The significance of the differences between means was verified using Tukey's t-test adjusting for multiple comparisons. The relationship between the identified polymorphisms and the level of gene expression in the uterus was verified taking into account the phase of the oestrus cycle in the gilts from crossbred group. The analysis of the relationship between the identified polymorphisms and the level of reproduction traits was carried out separately on the crossbred and the purebred groups. Additionally the relationship between *OPN* and *AREG* polymorphisms and expression levels of the selected genes by Spearman's rank correlation coefficient was estimated.

Results

Gene transcript expression in the uterine endometrium of sows in the luteal (L) and follicular (F) phases

The results of the mRNA analyses for 12 genes (*AREG*, *FABP3*, *IL1A*, *IL1B*, *ITGAV*, *ITGB3*, *NMB*, *OPN*, *PPARG*, *RBP4*, *S100A1*, *SELL*) in the uterine endometrium of 71 sows (cross-breed) in the luteal (46) and follicular (25) phases are shown in the graph below (Figure 1). Eight out of 12 genes, including *AREG*, *FABP3*, *IL1A*, *ITGAV*, *ITGB3*, *NMB*, *OPN*, and *RBP4*, showed higher expression at the mRNA level in the luteal phase. Higher expression in the follicular phase was showed by the *IL1B*, *PPARG*, *S100A8* and *SELL* genes.

For each gene, the differences between expression in the luteal and follicular phases were statistically significant at $P < 0.05$.

Figure 1. Mean and standard error (SE) values for relative expression at the mRNA level of genes encoding *AREG*, *FABP3*, *IL1A*, *IL1B*, *ITGAV*, *ITGB3*, *NMB*, *OPN*, *PPARG*, *RBP4*, *S100A8* and *SELL* in uterus of sows in the luteal and follicular phases

The correlation between transcript expression values of selected genes in uterus of gilts in the luteal and follicular phases and reproductive traits

The Pearson correlation coefficients presented in Table 3 indicate a significant negative relationship between the expression of the *OPN*, *AREG* and *SELL* genes in the luteal phase with the number of live-born and weaned piglets. Similar relationships were also found for *OPN* and *AREG* in the follicular phase (Table 4). At the same time, a mutual correlation of selected genes was identified. The results indicate high correlations between the expression of *FABP3*, *IL1A*, *IL1B*, *ITGAV*, *ITGB3*, *NMB*, *PPARG*, *RBP4* and *S100A8* in the luteal phase. Similarly, correlations of expression in the follicular phase were found mainly between *FABP3* and *IL1B*. In contrast, correlations between *OPN* and *AREG* expression levels were observed in both phases.

Polymorphism of selected genes

For the polymorphisms analysis, two genes encoding osteopontin *OPN* and amphiregulin *AREG* were selected based on previous research (Korwin-Kossakowska et al., 2013). There were also genes whose expression levels correlated with litter size in pigs. Screening tests carried out on the crossbred animals identified 13 polymorphic sites in the 2 genes. Of these thirteen polymorphisms 6 were considered for further analysis: four in the *OPN* gene (OPNe6_Knoll, OPNp3_617, OPNe6_462, OPNe7_603) and two in the *AREG* gene (AREGe2_317, AREGe3_561; Table 5). Seven of these polymorphisms were excluded from further analysis: one was a microsatellite sequence, while five located in the exons caused no amino acid changes of the protein and one turned out to be insufficiently polymorphic, which prevented their use for further association analyses.

Table 3. Pearson correlation coefficients between the transcript expression of *OPN*, *AREG* and other genes in the luteal phase with the number of live born and weaned piglets

Gen/trait	FABP3	IL1A	IL1B	ITGAV	ITGB3	NMB	PPARG	RBP4	S100A8	SELL	OPN	AREG
NBA	0.24	0.18	0.04	0.07	0.13	0.01	-0.08	0.13	-0.12	-0.38*	-0.44*	-0.53*
NPW	0.12	0.08	-0.03	-0.05	0.05	-0.10	-0.16	0.01	-0.14	-0.40*	-0.46*	-0.55*
FABP3	1	0.92*	0.80*	0.94*	0.87*	0.92*	0.77*	0.97*	0.50*	0.17	-0.09	-0.17
IL1A	0.92*	1	0.91*	0.84*	0.90*	0.85*	0.67*	0.94*	0.44*	0.15	-0.07	-0.17
IL1B	0.80*	0.91*	1	0.78*	0.90*	0.84*	0.70*	0.88*	0.53*	0.21	-0.08	-0.16
ITGAV	0.94*	0.84*	0.78*	1	0.89*	0.98*	0.91*	0.96*	0.67*	0.33**	-0.07	-0.14
ITGB3	0.87*	0.90*	0.90*	0.89*	1	0.91*	0.84*	0.92*	0.71*	0.35**	-0.09	-0.18
NMB	0.92*	0.85*	0.84*	0.98*	0.91*	1	0.93*	0.96*	0.70*	0.35**	-0.07	-0.14
PPARG	0.77*	0.67*	0.70*	0.91*	0.84*	0.93*	1	0.83*	0.88*	0.52*	-0.05	-0.11
RBP4	0.97*	0.94*	0.88*	0.96*	0.92*	0.96*	0.83*	1	0.58*	0.24	-0.08	-0.17
S100A8	0.50*	0.44*	0.53*	0.67*	0.71*	0.70*	0.88*	0.58*	1	0.69*	0.02	-0.05
SELL	0.17	0.15	0.21	0.33**	0.35**	0.35**	0.52*	0.24	0.69*	1	0.69*	0.60*
OPN	-0.09	-0.07	-0.08	-0.07	-0.09	-0.07	-0.05	-0.08	0.02	0.69*	1	0.92*
AREG	-0.17	-0.17	-0.16	-0.14	-0.18	-0.14	-0.11	-0.17	-0.05	0.60	0.92	1

*P≤0.01, **P<0.05.

NBA – number of live born piglets, NPW – number of weaned piglets.

Table 4. Pearson correlation coefficients between the transcript expression of *OPN*, *AREG* and other genes in the follicular phase with the number of live born and weaned piglets

Gene/trait	FABP3	IL1A	IL1B	ITGAV	ITGB3	NMB	PPARG	RBP4	S100A8	SELL	OPN	AREG
NBA	-0.02	0.08	-0.12	0.08	0.12	-0.09	-0.22	0.24	-0.20	-0.25	-0.56*	-0.39*
NPW	0.00	0.09	-0.16	0.02	0.01	-0.07	-0.22	0.28	-0.21	-0.28	-0.61*	-0.47*
FABP3	1	0.91*	0.75*	0.70*	0.51*	0.98*	0.47*	0.80*	0.55*	0.44*	-0.16	-0.26
IL1A	0.91*	1	0.61*	0.76*	0.48*	0.92*	0.23	0.76*	0.33*	0.22	-0.16	-0.12
IL1B	0.75*	0.61*	1	0.80*	0.38	0.70*	0.71*	0.42**	0.75*	0.85*	-0.18	-0.08
ITGAV	0.70*	0.76*	0.80*	1	0.33	0.67*	0.27	0.45**	0.36	0.46	-0.17	0.08
ITGB3	0.51*	0.48*	0.38*	0.33	1	0.51*	0.19	0.37	0.25	0.19	-0.17	-0.21
NMB	0.98*	0.92*	0.70*	0.67*	0.51*	1	0.44*	0.72*	0.53*	0.39	-0.11	-0.20
PPARG	0.47*	0.23*	0.71*	0.27	0.19	0.44*	1	0.13	0.99*	0.92*	-0.09	-0.18
RBP4	0.80*	0.76*	0.42*	0.45**	0.37	0.72*	0.13	1	0.20	0.07	-0.21	-0.33
S100A8	0.55*	0.33*	0.75*	0.36	0.25	0.53*	0.99*	0.20	1	0.92*	-0.11	-0.15
SELL	0.44*	0.22	0.85*	0.46	0.19	0.39	0.92*	0.07	0.92*	1	-0.10	-0.07
OPN	-0.16	-0.16	-0.18	-0.17	-0.17	-0.11	-0.09	-0.21	-0.11	-0.10	1	0.73*
AREG	-0.26	-0.12	-0.08	0.08	-0.21	-0.20	-0.18	-0.33	-0.15	-0.07	0.73*	1

* $P \leq 0.01$, ** $P \leq 0.05$.

NBA – number of live born piglets, NPW – number of weaned piglets.

Table 5. Polymorphism of the *OPN* and *AREG* genes. Tested fragments of genes

Gene	Site	Fragment	Mutation	Amino acid sequence
<i>OPN</i>	promoter	OPNp1_2	microsatellite	–
	promoter*	OPNp3_617	–617G<A	(transcription factor Tst-1)
	exon 1	OPNe1_1	–	–
	exon 6*	OPNe6_462	462C<A	synonymous substitution / RGD sequence
	intron 6*	OPNe6_Knoll	in/del	SINE
	exon 7*	OPNe7_603	603 A<G	synonymous substitution
	exon 7	OPNe7_2	–	–
<i>AREG</i>	exon 1	AREGe1_1	–	–
	exon 2*	AREGe2_317	317A<G	S114P
	exon 3*	AREGe3_561	561A<T	–
	exon 4	AREGe4_1	–	–
	exon 5	AREGe5_1	–	–
	exon 6	AREGe6_1	–	–

*The polymorphism taken for further analysis.

***OPN* polymorphism**

Within the DNA sequence encoding osteopontin, four polymorphic sites in the promoter, exon 6, intron 6 and exon 7 were selected for further analysis. The OPNp3_617 mutation in the *OPN* promoter region, caused by an G to A substitution, is located at the potential binding site of the Tst-1 transcription factor. In the analysed animal population three genotypes were identified: AA, AG and GG. The OPNe6_462 mutation in exon 6 is located in the highly conserved RGD region (amino acid sequence Arginine-Glycine-Asparagine) (Figure 2). In the analysed material, two alleles A and C and three genotypes were identified. In exon 7 a synonymous mutation OPNe7_603 was found. The G to A substitution generated three genotypes. The polymorphic sites detected in the gene sequence did not change the amino acid sequence of the protein as observed also earlier (Korwin-Kossakowska et al., 2013).

Figure 2. The electropherogram fragment of exon 6 sequences of the gene encoding osteopontin with the indication of the mutation site

Initially in intron 6 the polymorphism called OPNe6_Knoll, related to the insertion/deletion of the sequenced fragment was detected. Based on the methodology developed by the literature (Knoll et al., 1999), primers were designed for fragments of 498 or 193 bp allowing genotyping. As a result of the analysis of genotypes within this mutation, three different genotype forms of AA, AB, and BB were found, where the A allele is a wild type, while the B allele form is deleted with the SINE (short interspersed nuclear element) fragment.

***AREG* polymorphism**

Within the DNA sequence encoding amphiregulin, two polymorphic sites in exons 2 and 3 were selected for further analysis. The conversion of A<G in position 317 (AREGe2_317) caused a change in the amino acid sequence (S114P), while in fragment AREGe3_561, the conversion of A<T did not change the amino acid sequence of the protein. Two alleles were identified in *AREG* exon 2 – A and G and in *AREG* exon 3 – A and T, respectively.

Statistical analysis

Validation of potential biomarkers requires the use of different research groups (Pierzchała et al., 2014), therefore the first stage of the analysis included crossbred animals, while the next stage consisted in the verification of polymorphisms of the examined genes on a larger number of purebred PLW pigs (n=328). The highest heterozygosity was observed for polymorphisms in OPNe6_Knoll, OPNp3_617 and AREGe2_317 (Table 6). The lowest heterozygosity was found in the AREGe3_561 locus, which indicates low variability within this polymorphic site. The observed low frequency of the A allele for OPNe7_603 and AREGe3_561 in the studied animals also influenced the lower heterozygosity. Therefore, these two polymorphisms (OPNe7_603, AREGe3_561) were not taken into account for association analyses. The polymorphism index (PIC) was above 0.3 for OPNe6_Knoll, OPNp3_617 and AREGe2_317, whereas the AREGe3_561 polymorphism was characterized by the lowest value.

The consistency of the genotype frequency in the analysed population with the Hardy–Weinberg's law is presented in Table 6 A.

Table 6. Frequency of alleles, heterozygosity and PIC value of analysed loci in investigated populations – crossbred and purebred animals jointly

Locus	Allele	Frequency %	Expected heterozygosity	Heterozygosity (unbiased)	PIC
1	2	3	4	5	6
OPNe6_Knoll	A	39.94	0.479755	0.4805	0.36467
	B	60.06			
OPNp3_617	A	46.04	0.496858	0.4976	0.37342
	G	53.96			
OPNe6_462	A	23.63	0.360904	0.3615	0.29578
	C	76.37			

Table 6 – contd.

1	2	3	4	5	6
OPNe7_603	A	06.10	0.114515	0.1147	0.10796
	G	93.90			
AREGe2_317	A	53.05	0.498141	0.4989	0.37407
	G	46.95			
AREGe3_561	A	0.76	0.015128	0.0152	0.01501
	T	99.24			

PIC – polymorphism information content.

Table 6 a. Hardy–Weinberg equilibrium test of analysed gene polymorphisms in groups of animals (Genepop 4.7.0)

Group		Locus					
		OPNe6-Knoll	OPNp3_617	OPNe6_462	OPNe7_603	AREGe2_317	AREGe3_561
Group I N=71	L phase	ns	*	ns	***	*	ns
	F phase	ns	ns	ns	ns	**	ns
Group II N=328		ns	***	***	***	***	ns

ns=non-significant, *P<0.05, **P<0.01, ***P<0.001.

Relationship between gene polymorphisms and transcript expression levels in the uterus

Analysis of the relationship between the transcript expression levels of the 12 selected genes and six polymorphic sites in the *OPN* gene (OPNe6_Knoll, OPNp3_617, OPNe6_462, OPNe7_603) and *AREG* (AREGe2_317, AREGe3_561) showed a significant ($P<0.05$) relationship between the *ITGB3* transcript expression levels and the OPNe7_603 genotype, while at $P\leq 0.01$ it was between the *IL1A* transcript expression levels and the OPNe7_603 genotype (Table 7). Very close to significant results were recorded for the OPNe3_617 genotype and *IL1A* expression and OPNe7_603 genotype and *IL1B* expression. Additionally the relationship between expression of selected candidate genes and *OPN* and *AREG* polymorphism separately in luteal and follicular phase by Spearman's rank correlation coefficient were estimated. This analysis indicated significant ($P<0.05$) relationship between the *FABP3* transcript expression levels and the OPNe6_462 genotype and very close to significant results were recorded for the OPNe6_462 genotype and *RBP4* transcript expression (Tables 9 a). In the group of sows in luteal phase very close to significant results were recorded for the OPNe6_462 genotype and *ITGB3* transcript expression. (Table 9 b). However, no relation was observed between the polymorphic sites of the *OPN* and *AREG* genes and expression of their transcripts (Tables 7, 8).

Table 7. Relationship between *OPN* and *AREG* polymorphisms and transcript expression levels of the *AREG*, *FABP3*, *IL1A*, *IL1B*, *ITGAV* and *ITGB3* genes

Locus	DF	<i>AREG</i>		<i>FABP3</i>		<i>IL1A</i>		<i>IL1B</i>		<i>ITGAV</i>		<i>ITGB3</i>	
		F	p	F	p	F	p	F	p	F	p	F	p
OPNe6_Knoll	2	0.00	0.9950	0.85	0.4317	0.58	0.5623	0.13	0.8788	1.15	0.3243	0.12	0.8894
OPNp3_617	2	0.29	0.7458	2.82	0.0675	3.05	0.0550	2.91	0.0626	2.50	0.0907	2.08	0.1340
OPNe6_462	2	0.02	0.9837	2.87	0.0647	2.86	0.0652	0.63	0.5385	2.49	0.0917	1.94	0.1529
OPNe7_603	2	0.35	0.7045	1.82	0.1706	5.65	0.0057	3.02	0.0563	1.16	0.3218	3.51	0.0363
AREGe2_317	2	0.44	0.6488	1.87	0.1637	2.10	0.1317	0.76	0.4710	2.03	0.1399	1.76	0.1811
AREGe3_561	1	0.06	0.8093	0.34	0.5643	0.24	0.6240	0.09	0.7598	0.24	0.6292	0.06	0.8130

F – F statistic, P – level of significance, DF – degrees of freedom.

Table 8. Relationship between *OPN* and *AREG* polymorphisms and transcript expression levels of the *NMB*, *OPN*, *PPARG*, *RBP4*, *SI00A8* and *SELL* genes

Locus	DF	<i>NMB</i>		<i>OPN</i>		<i>PPARG</i>		<i>RBP4</i>		<i>SI00A8</i>		<i>SELL</i>	
		F	p	F	p	F	p	F	p	F	p	F	p
OPNe6_Knoll	2	1.34	0.2696	0.19	0.8253	1.71	0.1894	0.87	0.4247	1.03	0.3620	0.97	0.3865
OPNp3_617	2	2.87	0.0645	0.13	0.8821	0.80	0.4533	2.45	0.0953	0.83	0.4412	1.24	0.2975
OPNe6_462	2	1.82	0.1716	0.08	0.9267	2.21	0.1187	1.88	0.1613	2.83	0.0673	1.61	0.2087
OPNe7_603	2	1.11	0.3358	0.05	0.9546	0.27	0.7675	1.98	0.1476	0.05	0.9486	0.10	0.9061
AREGe2_317	2	1.53	0.2242	0.08	0.9247	0.93	0.3985	2.04	0.1391	1.17	0.3184	0.72	0.4925
AREGe3_561	1	0.14	0.7064	0.03	0.8636	0.07	0.7881	0.40	0.5277	0.04	0.8336	0.00	0.9489

F – F statistic, P – level of significance, DF – degrees of freedom.

Table 9 a. Relationship between *OPN* and *AREG* polymorphisms and transcript expression levels of the selected genes in follicular phase estimated by Spearman's rank correlation (N=25)

		<i>AREG</i>	<i>FABP3</i>	<i>IL1A</i>	<i>IL1B</i>	<i>ITGAV</i>	<i>ITGB3</i>	<i>NMB</i>	<i>PPARG</i>	<i>RBP4</i>	<i>S100A8</i>	<i>SELL</i>	<i>OPN</i>
OPNe6Knoll	Correlation coefficient	-0.16	-0.14	-0.19	-0.04	-0.15	0.05	-0.12	0.03	-0.15	-0.13	-0.07	-0.30
	P-value	0.43	0.52	0.35	0.84	0.49	0.82	0.58	0.88	0.48	0.54	0.73	0.14
OPNp3_617	Correlation coefficient	-0.18	-0.12	-0.11	-0.17	-0.06	0.11	-0.11	0.19	-0.07	-0.25	-0.07	-0.09
	P-value	0.38	0.58	0.59	0.42	0.76	0.60	0.59	0.36	0.73	0.23	0.74	0.68
OPNe6_462	Correlation coefficient	-0.38	0.43	0.33	0.13	0.09	0.16	0.27	0.17	0.39	0.19	0.06	0.23
	P-value	0.06	0.03	0.11	0.54	0.65	0.45	0.19	0.41	0.05	0.35	0.77	0.27
OPNe7_603	Correlation coefficient	0.03	0.25	0.20	0.25	0.25	0.23	0.14	0.20	0.23	0.06	0.31	0.34
	P-value	0.89	0.22	0.34	0.22	0.22	0.28	0.50	0.34	0.28	0.79	0.13	0.10
AREGe2_317	Correlation coefficient	-0.06	-0.08	-0.06	0.03	-0.23	-0.02	-0.09	-0.20	-0.08	-0.35	-0.14	-0.19
	P-value	0.79	0.71	0.79	0.87	0.28	0.91	0.67	0.33	0.71	0.09	0.52	0.36
AREGe3_561	Correlation coefficient	0.12	0.18	0.12	0.23	0.18	0.17	0.17	0.02	0.18	0.08	0.14	0.24
	P-value	0.35	0.45	0.35	0.14	0.45	0.48	0.40	0.83	0.45	0.83	0.27	0.16

Correlation is significant at the P<0.05 level.

Table 9 b. Relationship between *OPN* and *AREG* polymorphisms and transcript expression levels of the selected genes in luteal phase estimated by Spearman's rank correlation coefficient (N=46)

		<i>AREG</i>	<i>FABP3</i>	<i>IL1A</i>	<i>IL1B</i>	<i>ITGAV</i>	<i>ITGB3</i>	<i>NMB</i>	<i>PPARG</i>	<i>RBP4</i>	<i>SI0048</i>	<i>SELL</i>	<i>OPN</i>
OPNe6Knoll	Correlation coefficient	0.14	0.03	-0.05	0.01	0.02	0.12	-0.07	-0.02	0.02	0.02	0.14	0.001
	P-value	0.35	0.87	0.75	0.93	0.89	0.42	0.66	0.89	0.89	0.89	0.35	0.99
OPNp3_617	Correlation coefficient	0.06	-0.15	-0.08	-0.06	-0.07	0.07	-0.01	-0.10	-0.16	-0.11	-0.03	0.01
	P-value	0.68	0.32	0.59	0.67	0.65	0.64	0.94	0.53	0.30	0.47	0.82	0.95
OPNe6_462	Correlation coefficient	-0.09	-0.19	-0.22	-0.18	-0.24	-0.29	-0.17	-0.24	-0.15	0.02	-0.13	-0.10
	P-value	0.57	0.20	0.15	0.24	0.11	0.05	0.25	0.11	0.32	0.90	0.41	0.52
OPNe7_603	Correlation coefficient	0.03	0.00	0.03	-0.23	0.02	0.10	0.15	-0.13	0.06	-0.16	0.03	-0.06
	P-value	0.85	0.99	0.86	0.13	0.88	0.52	0.31	0.38	0.71	0.28	0.82	0.70
AREGe2_317	Correlation coefficient	-0.26	-0.10	0.08	0.13	-0.17	-0.16	-0.03	-0.07	-0.10	-0.02	-0.19	-0.22
	P-value	0.08	0.52	0.61	0.38	0.26	0.28	0.87	0.62	0.51	0.91	0.19	0.14
AREGe3_561	Correlation coefficient	0.15	0.18	0.15	0.23	0.12	0.11	0.11	0.18	0.12	0.03	0.17	0.24
	P-value	0.32	0.43	0.32	0.12	0.44	0.48	0.48	0.85	0.44	0.85	0.25	0.11

Correlation is significant at the P<0.05 level.

Table 10. Relationship between *OPN* and *AREG* genotypes and number of piglets born alive in the litter for the crossbred group and the purebred group

NBA					
Locus	DF	PLW × PL (n=71)		PLW (n=328)	
		F	P	F	P
OPNe6_Knoll	2	0.09	0.9129	2.56	0.0790
OPNp3_617	2	0.99	0.3780	3.15	0.0441
OPNe6_462	2	0.56	0.5765	3.43	0.0335
OPNe7_603	2	0.48	0.6221	0.07	0.9325
AREGe2_317	2	0.55	0.5794	3.41	0.0342
AREGe3_561	1	0.14	0.7080	0.72	0.3982

DF – degrees of freedom, F – F statistic, P – level of significance.

The mean values and standard deviations of relative gene expression for the tested *OPN* genotypes in the luteal and follicular phases are presented in Supplement Tables 1 a, b and Supplement Tables 2 a, b, respectively, while the exact genotype and gene expression with respect to the oestrous cycle phase are presented in Supplement Tables 3 a, b.

Due to the very low frequencies of some genotypes, such polymorphic sites were excluded from these analyses.

Relationship between selected *OPN* and *AREG* genotypes and reproductive traits

Analysis of the relation between six polymorphic sites in the *OPN* (OPNe6_Knoll, OPNp3_617, OPNe6_462, OPNe7_603) and *AREG* (AREGe2_317, AREGe3_561) genes and reproductive traits showed a significant ($P < 0.05$) relation of the number of piglets born alive (NBA) with the OPNp3_617, OPNe6_462 and AREGe2_317 genotypes for the purebred sows (PLW). In the case of the crossbred sows (PLW × PL) no significant relationship was observed (Table 10). Similarly, no relationship was found between the *OPN* and *AREG* genotypes and the number of piglets weaned on day 21 (NPW) in any of the studied populations (Table 11).

Table 11. Relationship between *OPN* and *AREG* genotypes and number of piglets weaned in the litter for the crossbred group and the purebred group

NPW					
Locus	DF	PLW × PL (n=71)		PLW (n=328)	
		F	P	F	P
OPNe6_Knoll	2	0.07	0.9327	2.24	0.1081
OPNp3_617	2	1.13	0.3309	1.82	0.1643
OPNe6_462	2	0.23	0.7937	2.43	0.0900
OPNe7_603	2	0.18	0.8398	0.04	0.9572
AREGe2_317	2	1.10	0.3410	2.79	0.0632
AREGe3_561	1	0.01	0.9122	0.76	0.3840

DF – degrees of freedom, F – F statistic, P – level of significance.

In the case of OPNp3_617 polymorphism (Table 12), the largest number of piglets in the litter were AG heterozygotes, while the lowest number were GG homozygotes.

For OPNe6_462 (Table 12), the highest number of piglets in the litter were also AC heterozygotes, while the lowest number were AA homozygotes.

In the case of the AREGe2_317 amphiregulin genotype, the smallest number of piglets in the litter were GG homozygotes, while there were no significant differences between the other AA homozygotes and the AG heterozygotes (Table 12).

Table 12. Mean values and standard deviations of reproductive traits depending on the genotype of selected candidate genes in the purebred group (only significant polymorphisms)

Locus	Genotype	N	NBA		NPW	
			Mean	SD	Mean	SD
OPNp3_617	AA	44	10.82	1.79	10.34	1.54
	AG	214	11.15 a	1.26	10.55	1.29
	GG	70	10.70 b	1.43	10.23	1.34
OPNe6_462	AA	35	10.46 a	1.42	10.17	1.38
	AC	85	11.31 b	1.58	10.75	1.47
	CC	208	10.98	1.27	10.38	1.26
AREGe2_317	AA	60	10.93	1.21	10.40	1.15
	AG	228	11.13 a	1.42	10.55 a	1.37
	GG	40	10.45 b	1.32	9.98 b	1.39

Mean – mean value, N – number of observation, SD – standard deviation, NBA – number of piglets born alive, NPW – number of piglets weaned, a, b – means with different letters in columns differ at $P < 0.05$.

Discussion

The different level of expression in the uterus of cyclic sows results from the different role played by the products of the studied genes in the organism. The *AREG*, *FABP3*, *IL1A*, *IL1B*, *ITGAV*, *ITGB3*, *NMB*, *OPN*, *PPARG*, *RBP4*, *S100A1* and *SELL* genes form a functional network based on common biological processes, closely related to the level of mRNA expression, and are expressed in the endometrium of gilts in various moments of the oestrus cycle. The observed mutual correlation of the results of the expression of the studied genes (coexpression) is related to their participation in these processes. Our research shows that most of the investigated genes indicated the highest expression level in the endometrium of the uterus in cycling sows during the luteal phase except for four genes *IL1B*, *PPARG*, *S100A8*, and *SELL*. As for *PPARG*, the differences between the phases were not significant. Also, the literature data show that the expression of this protein is relatively stable in the cycle (Bogacka and Bogacki, 2011). As for the *IL1B*, *S100A8*, and *SELL* genes, their pro-inflammatory role is probably important and it is generally associated with the preparation of the uterine epithelium for pregnancy (Altmäe et al., 2010; Nair et al.,

2013; Pasetj et al., 1999; Rosen, 2004). The present study, by focusing on cycling sows, provides data showing that transcript expression (mRNA) of the most interesting genes *OPN* and *AREG* in the uterus increases in the luteal phase. These results find confirmation in studies conducted by other authors, e.g. Carson et al. (2002) showed osteopontin mRNA expression 12 times higher in the human endometrium between the early luteal (pre-receptive) and mid-luteal (receptive) phases. Similar results were also obtained by White et al. (2006), who analyzed gene expression in murine endometrium. For these animals, osteopontin is expressed both in the epithelium and leukocyte and macrophage immune cells (White et al., 2006). Expression of osteopontin in the surface epithelium distinguishes pigs from animals of a different type of placentation, e.g. goats and sheep, and also in humans, in whom this protein is expressed during pregnancy only in the glandular epithelium, which supplies the uterus with this protein as part of histotroph (Joyce et al., 2005).

The results of our analysis indicate that *AREG* mRNA expression in the luteal phase is almost twice as high as in the follicular phase. What may be the reason? The expression of *AREG* in the endometrium changes throughout the cycle; however, results of similar analyses, e.g. concerning humans are inconclusive. Only the results obtained by Gui et al. (2008) seem to confirm our results. Those authors stated that the expression of amphiregulin mRNA in the human endometrium was 32 times higher in the secretory (luteal) than in the proliferative (follicular) phase. The expression of amphiregulin was increased in the secretory phase of the human endometrium, which suggests that amphiregulin may play an important role in embryo implantation (Gui, 2008). These results were also confirmed by protein analysis. However, Ejlskjær et al. (2005) stated that these tendencies are rather opposite.

Our results related to the polymorphism of selected genes are also very interesting. The *OPN* gene is located on the long arm of the *Sus scrofa* chromosome 8 (SSC8q), composed of seven exons and six introns, which encoded a protein of 303 amino acids (Ensembl database: http://asia.ensembl.org/Sus_scrofa/Info/Index) and is structurally similar to that of other genes. But the difference lays in the promoter of this gene. There is no classic TATA box sequence, it seems that its functions are performed by the TTAAAA sequence. The presence in the promoter of this gene of a "potential early growth response element" sequence binding EGR-1 gene (a factor regulating gene transcription, also related to cell proliferation) suggests that the *OPN* gene may respond indirectly to growth factors through interaction with this gene (Zhang et al., 1992). In our work, the existence of new polymorphic sites in the promoter of the gene and coding region (exons 6 and exon 7) of the gene encoding osteopontin has also been demonstrated. The investigated mutation in the *OPN* promoter region – OPNp3_617 (the exchange of nucleotide G to A) is located at the potential binding site of the Tst-1 transcription factor. The other polymorphism in the osteopontin sequence – OPNe6_462 (the exon 6 mutation), although not causing the encoded amino acid to change, is located in the highly conserved region of RGD (RGD motif – peptide sequence Arg-Gly-Asp) that is involved in cell adhesion (Garlow et al., 2002). This sequence is recognized by more than half of known integrins and is the main system allowing cell adhesion and signaling necessary during pregnancy in the implantation process (Ruoslahti, 1996). The blockage of the RGD

region significantly reduced the adhesion effect of the trophoblast to the epithelium induced by osteopontin (Erikson et al., 2009). These processes may be reflected in the reproduction results. Osteopontin, by connecting to the integrin receptors present on both the trophoblast surface and the surface epithelium of the uterus, which is made possible by its RGD motif causes a reorganization of the cell cytoskeleton (White et al., 2005). It leads, among others, to the cell-cell and cell-ECM (extracellular matrix) adhesion, leukocyte chemotaxis, macrophage migration, and activation of mechanisms conditioning epithelial cell survival (Spencer et al., 2008).

The gene coding amphiregulin (*AREG*) is located on pig's chromosome 8, in the area of a QTL for uterine capacity (Kim et al., 2001). This gene consists of 6 exons and 5 introns and contains 10.3 kB. In humans two forms of the gene are found to be associated with the presence or absence of exon 5, coding the cytoplasmic domain (Kim et al., 2003). In recent research results, a lot has been written about the study of *AREG* gene polymorphism in the aspect of human diseases. Single nucleotide polymorphism in *AREG* gene has become a prognostic biomarker in locally advanced gastric cancer (GC) patients, also three single-nucleotide polymorphisms in the *AREG* gene region have been related with predicted disease control in the metastatic colorectal cancer (mCRC) patients (Wakatsuki et al., 2014; Sebio et al., 2014). Our research showed the existence of two, previously unidentified polymorphisms of this gene, in the exons 2 (317A <G) and 3 (561A <T). The conversion of A<G in position 317 (*AREGe2_317*) caused a change in the amino acid sequence (S114P), while in fragment *AREGe3_561*, the conversion of A<T did not change the amino acid sequence of the protein.

Individual variation in the level of gene expression, apart from environmental effects, may be due to genetic causes (Stranger et al., 2005) and is often associated with the regulation of polymorphic regions (Cheung et al., 2010). The effects of the *OPNe3_617* genotype on the *IL1A* expression level and the *OPNe7_603* genotype on the *IL1B* expression are close to the level of statistical significance, while the effects of the *OPNe7_603* genotype on the *IL1A* and *ITGB3* transcript expression are statistically significant. Also the estimation of correlation, separately in luteal and follicular phase by Spearman's rank correlation coefficient, between *OPN* polymorphism and *ITGB3* gene transcript expression, indicated the significant results. All these effects observed in the current study may suggest an association of osteopontin with the regulation of other genes, especially in the case of interleukins, as is also shown by the analysis of the network of links between genes. However, no effects of the polymorphism in the examined *OPN* and *AREG* genes on the level of their own expression neither in the luteal nor in the follicular phase were observed. An opposite relationship was observed by Korwin-Kossakowska (2013), but in that case, the luteal and follicular phases were considered jointly. Although the relationship between the genotype of osteopontin and its expression was observed by other authors, it concerned the sequence of the intron. SNP in the first intron of osteopontin influenced its expression by activating alternative splicing (Muráni et al., 2009).

The results of our research allowed us to identify significant relationships between the transcript expression of *OPN* and *AREG* and reproductive traits NBA and NPW in luteal and also in follicular phases. The significant relationships between *OPN* and *AREG* genotypes and the number of piglets born alive in the litter were

also observed in our research. This relation indicates the involvement of the studied genes in the regulation of processes related to the establishment and maintenance of pregnancy. The OPNp3_617 and OPNe6_462 genotypes significantly influenced the number of piglets born, with the highest value in both cases being recorded for heterozygotes. A similar effect was observed in boars and was associated with sperm quality (Lin et al., 2006). However, the dependencies indicated by us were shown only in the case of the purebred sow population. For the analyzed hybrid crosses, similar relationships were statistically non-significant. This may be due either to a small number of observations or crossbreeding. The reason for this may also be connected with small differences between the breeds, although the scale effect seems more likely (DeWinter, 2013). On the other hand, the deletion-insertion polymorphism associated with the occurrence of the SINE sequence (PRE-1) (Knoll et al., 1999), which was associated with the mass of the uterus in the Polish landrace breed (Kapelański et al., 2013), the size of litter in Tibetan pigs (Niu et al., 2008), and the number of weaned piglets of the Polish synthetic line 990 (Korwin-Kossakowska et al., 2002), in the currently studied populations showed no relationship with the number of born or weaned piglets. The performed analyses clearly support the position of osteopontin as a candidate gene associated with prenatal survival and litter size (King et al., 2003).

However, in the case of the amphiregulin coding gene, the genotype associated with the polymorphic site in exon 2 (AREGe2_317) had a significant effect on reproductive traits. The lowest number of piglets in the litter was recorded for the GG animals compared to the AG heterozygotes. In another published study the polymorphism of the same gene referred to as the A1A1 and A1A2 genotypes, and associated with the C → T mutation was correlated with the number of piglets born alive in the 2th and the 4th successive litters (Mucha et al., 2013). In the Meishan pigs, the A1 allele had a negative effect on the number of piglets born alive (Jiang et al., 2002). The influence of amphiregulin polymorphism on litter size may result from its biological role as one of the epidermal growth factors and may be related both with the participation in implantation (Das et al., 1995) and in ovulation (Freimanna et al., 2004).

Conclusions

The present transcript analyses results confirmed us to correct selection of the genes which could be involved in the regulation of reproductive processes in sows. Two of the chosen genes, coding osteopontin and amphiregulin, respectively, showed the significant relationship with traits related to litter size at both the level of mRNA expression and gene polymorphism. Two polymorphic sites in the *OPN* gene – in the promoter and exon 6, as well as one in the *AREG* gene in exon 2 – indicated a relationship with litter size in pure-breed sows. In all these cases heterozygous sows produced more numerous litters than the others. On the other hand, the *OPN* polymorphisms in exon 7 and exon 6 were statistically significantly related to the uterine mRNA expression level of the other investigated genes. Thus, the obtained results seem to indicate osteopontin and amphiregulin as candidate genes/markers for further swine selection.

Declaration of interests

The authors declare that they do not have any conflict of interest.

Table 1 a (Supplement). Mean values and standard deviations of relative gene transcript expression in the luteal phase for the investigated *OPN* genotypes

Phase of oestrous cycle	Locus	Genotype	N	Gene expression											
				AREG		FABP3		IL1A		IL1B		ITGAV		ITGB3	
				M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
L	OPNe6_Knoll	AA	4	0.69	1.08	3.48	6.23	3.76	7.06	2.19	3.77	4.95	9.21	3.21	5.64
		AB	26	1.25	3.44	0.91	1.13	0.77	1.03	0.67	0.92	1.26	2.25	1.04	1.99
		BB	16	1.17	1.74	1.04	1.37	1.41	2.89	0.90	1.84	0.81	1.04	1.56	2.12
	OPN p3_617	AA	5	1.03	1.56	1.54	1.80	1.41	1.97	1.20	1.82	2.51	4.70	2.50	4.31
		AG	31	1.34	3.32	1.33	2.44	1.49	3.20	0.95	1.87	1.56	3.41	1.41	2.53
		GG	10	0.72	0.61	0.54	0.52	0.44	0.37	0.50	0.46	0.47	0.38	0.88	0.71

L – luteal phase, N – number of observations, M – mean value, SD – standard deviation.

Table 1 b (Supplement). Mean values and standard deviations of relative gene transcript expression in the luteal phase for the investigated *OPN* genotypes

Phase of oestrous cycle	Locus	Genotype	N	Gene expression											
				NMB		OPN		PPARG		RBP4		S100A8		SELL	
				M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
L	OPNe6_Knoll	AA	4	4.80	8.38	0.22	0.40	3.10	5.80	3.71	6.92	0.35	0.59	0.35	0.50
		AB	26	0.97	2.06	2.45	11.86	0.88	2.48	0.93	1.25	0.30	0.72	1.18	2.92
		BB	16	0.75	1.04	0.07	0.12	0.39	0.35	1.02	1.42	0.16	0.14	0.39	0.28
	OPN p3_617	AA	5	2.47	4.64	0.09	0.15	2.88	5.60	1.88	2.45	0.99	1.58	2.39	4.75
		AG	31	1.26	3.11	2.03	10.87	0.73	2.08	1.32	2.63	0.17	0.22	0.74	1.97
		GG	10	0.51	0.29	0.25	0.67	0.45	0.48	0.51	0.43	0.16	0.13	0.33	0.25

L – luteal phase, N – number of observations, M – mean value, SD – standard deviation.

Table 2 a (Supplement). Mean values and standard deviations of relative gene transcript expression in the follicular phase for the investigated *OPN* genotypes

Phase of oestrous cycle	Locus	Genotype	N	Gene expression											
				AREG		FABP3		IL1A		IL1B		ITGAV		ITGB3	
				M	SD	M	SD	M	SD	Mn	SD	M	SD	M	SD
P	OPNe6_Knoll	AA	6	1.22	1.26	0.44	0.33	0.40	0.61	0.35	0.39	0.20	0.28	0.08	0.07
		AB	10	0.47	0.60	1.08	1.83	0.88	1.63	1.85	2.93	0.26	0.37	0.37	0.42
		BB	9	0.55	0.53	0.37	0.41	0.22	0.33	1.10	2.10	0.17	0.38	0.21	0.38
	OPN p3_617	AA	4	1.65	1.43	1.84	2.71	1.97	2.35	3.48	3.56	0.78	0.56	0.36	0.46
		AG	15	0.47	0.53	0.36	0.33	0.18	0.22	0.44	0.47	0.07	0.10	0.17	0.30
		GG	6	0.56	0.47	0.67	0.94	0.45	0.53	1.67	3.16	0.19	0.18	0.35	0.44
	OPN e6_462	AA	3	1.35	1.92	0.85	1.44	0.33	0.54	2.75	4.63	0.17	0.29	0.15	0.25
		AC	6	0.89	0.54	0.16	0.19	0.27	0.50	0.42	0.43	0.13	0.11	0.27	0.46
		CC	16	0.47	0.55	0.83	1.38	0.67	1.32	1.23	2.12	0.26	0.41	0.25	0.35

P – follicular phase, N – number of observations, M – mean value, SD – standard deviation.

Table 2 b (Supplement). Mean values and standard deviations of relative gene transcript expression in the follicular phase for the investigated *OPN* genotypes

Phase of oestrous cycle	Locus	Genotype	N	Gene expression											
				NMB		OPN		PPARG		RBP4		S100A8		SELL	
				M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
P	OPNe6_Knoll	AA	6	0.13	0.17	0.54	1.00	0.15	0.20	0.57	0.37	0.92	1.15	0.17	0.10
		AB	10	1.27	2.62	0.05	0.14	2.61	6.35	0.65	1.10	4.80	11.37	2.26	5.54
		BB	9	0.10	0.13	0.16	0.43	0.28	0.42	0.65	0.88	0.64	0.75	1.01	2.32
	OPN p3_617	AA	4	2.26	4.03	0.64	1.27	1.02	1.63	1.18	1.72	3.44	4.51	2.56	3.25
		AG	15	0.11	0.13	0.06	0.12	0.19	0.34	0.58	0.69	0.48	0.63	0.19	0.25
		GG	6	0.61	1.17	0.29	0.52	3.75	8.18	0.38	0.32	6.39	14.49	3.27	7.16
	OPN e6_462	AA	3	1.00	1.71	0.85	1.47	6.82	11.79	0.22	0.32	12.06	20.68	6.02	10.25
		AC	6	0.09	0.10	0.22	0.52	0.14	0.13	0.29	0.31	0.45	0.75	0.23	0.26
		CC	16	0.68	2.04	0.08	0.15	0.51	0.89	0.83	1.01	1.27	2.46	0.83	1.80

P – follicular phase, N – number of observations, M – mean value, SD – standard deviation.

Table 3 a (Supplement). Relationship between *OPN* polymorphism and relative gene transcript expression

Phase of oestrous cycle	Locus	DF	Sum of squares	Gene expression	Test F-value	P-value
L	OPNe6_Knoll	2	28.02	<i>FABP3</i>	4.26	0.02
		2	73.12	<i>ITGAV</i>	4.58	0.02
		2	72.65	<i>NMB</i>	5.43	0.01
		2	38.78	<i>PPARG</i>	3.51	0.04
	OPNp3_617	2	33.80	<i>RBP4</i>	4.17	0.02
		2	24.29	<i>FABP3</i>	3.70	0.04
		2	35.00	<i>IL1A</i>	3.39	0.05
		2	44.42	<i>PPARG</i>	4.02	0.03
	OPNp3_617	2	27.36	<i>RBP4</i>	3.38	0.05
		2	3.38	<i>S100A8</i>	5.29	0.01
		2	12.02	<i>IL1A</i>	6.97	0.01
		2	1.75	<i>ITGAV</i>	15.39	0.00
P	OPNe6_462	2	3.99	<i>AREG</i>	6.27	0.01
		2	181.09	<i>PPARG</i>	11.37	0.00
		2	515.43	<i>S100A8</i>	8.53	0.00
		2	134.79	<i>SELL</i>	7.65	0.01
	OPNp3_617	2	3.77	<i>AREG</i>	5.92	0.01
		2				
		2				
		2				

L – luteal phase, P – follicular phase, DF – degrees of freedom.

Table 3 b (Supplement). Relationship between relative gene transcript expression and *OPN* genotype by Tukey's test at $P<0.05$

Phase of oestrous cycle	Locus	Gene expression	Genotype comparison	Difference	PU-95% lower	PU-95% upper	Level of significance	
L	1	2	3	4	5	6	7	8
		OPNe6_Knoll						
			<i>FABP3</i>	AA - AB	2.56	0.18	4.95	P<0.05
			<i>ITGAV</i>	AA - BB	4.13	0.26	8.00	P<0.05
			<i>NMB</i>	AA - AB	3.83	0.42	7.23	P<0.05
			<i>NMB</i>	AA - BB	4.05	0.50	7.59	P<0.05
			<i>RBP4</i>	AA - AB	2.79	0.14	5.44	P<0.05
			<i>SI0048</i>	AA - AG	0.82	0.15	1.49	P<0.05
		OPNp3_617	<i>SI0048</i>	AA - GG	0.83	0.07	1.59	P<0.05
			<i>IL1A</i>	AA - GG	4.31	0.27	8.34	P<0.05
			<i>IL1A</i>	AA - AG	5.16	0.08	10.24	P<0.05
			<i>IL1B</i>	AA - GG	3.26	0.65	5.87	P<0.05
			<i>IL1A</i>	AA - AG	1.79	0.44	3.15	P<0.05
		OPNp3_617	<i>IL1B</i>	AA - AG	3.04	0.16	5.92	P<0.05
			<i>ITGAV</i>	AA - GG	0.59	0.19	0.99	P<0.05
			<i>ITGAV</i>	AA - AG	0.71	0.36	1.06	P<0.05
			<i>PPARG</i>	AG - GG	-3.56	-7.10	-0.02	P<0.05
			<i>AREG</i>	AA - GG	1.09	0.15	2.04	P<0.05
			<i>AREG</i>	AA - AG	1.18	0.36	2.00	P<0.05
			<i>PPARG</i>	AA - CC	6.31	1.70	10.92	P<0.05
		OPNe6_462	<i>PPARG</i>	AA - AC	6.69	1.50	11.87	P<0.05
			<i>SI0048</i>	AA - CC	10.79	1.81	19.78	P<0.05
P								

Table 3 b (Supplement) – contd.

1	2	3	4	5	6	7	8
		<i>ST0048</i>	AA - AC	11.62	1.52	21.72	P<0.05
		<i>SELL</i>	AA - CC	5.19	0.34	10.04	P<0.05
		<i>SELL</i>	AA - AC	5.79	0.34	11.25	P<0.05

L – luteal phase, P – follicular phase, PU-95% lower – lower confidence limit, PU-95% upper – confidence interval (limit).

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