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POLYMORPHISMS OF THE OSTEOPONTIN GENE AND LEVEL OF ITS EXPRESSION IN THE REPRODUCTIVE TRACT OF SOWS*

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Abstract

The aim of the study was to find osteopontin gene (*OPN*) polymorphisms as potential mutations affecting the expression level of genes in the ovaries, uterus and oviduct of sows. The material consisted of 71 F_1 sows (Polish Large White × Polish Landrace). In the first stage several polymorphisms in the promoter region, intron 6, exon 6 and 7 of the *OPN* gene were found. The parameters estimated were the frequency of alleles and genotypes, observed heterozygosity and gene diversity, PIC, and chi² factors. Chi² values allow for assessment of genetic equilibrium in the population. Thus, the loci OPNp3-4 and OPNe6-1 were in genetic disequilibrium while locus OPNe6-Knoll showed genetic equilibrium. Also real-time PCR analysis to determine the expression dynamics of the *OPN* gene in examined tissues was performed in relation to "housekeeping" genes. A comparison was made for relative expression in different tissues and different mutations. The highest expression pattern was observed in the oviduct. Based on the novel polymorphisms a significant correlation between the *OPN* genotype and *OPN* expression (mRNA) level in the ovary, oviduct, uterine body and uterine horn was observed. In the second stage, the levels of expression of the *OPN* gene in individual tissues, traits of reproductive performance and reproductive tract traits of sows were also compared. The expression levels in the uterine body and oviduct were related to the age of mating, cervical length, litter weight at birth, number of active nipples, age at slaughter and body weight at mating.

Key words: sow, reproduction, *OPN* polymorphism, gene expression

The efficiency of swine production depends on a variety of factors, of which reproductive traits form an important part. However, the genetic progress in improving these traits seems small. The polygenic nature and the low heritability of the sow's reproductive traits are an objective obstacle. Against the background of the results

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of earlier research, the need to develop an unconventional way of increasing the efficiency of swine reproduction is becoming increasingly common. One way to achieve this goal could be to use some basic techniques of molecular genetics, identifying the DNA polymorphism and mechanisms of gene expression in the tissues of the reproductive system (Tuggle, 2004). In recent years, transcriptase analysis has become a very popular molecular examination of living organisms, which enables understanding gene expression profiles in cells. Differences in the processes related to metabolism in reproductive system tissues are the result of varied expression levels of many genes. Therefore, gene expression profiles in samples taken from sow reproductive tissues such as uterus, ovary and oviduct could provide valuable information on the potential association with the subsequent implantation of embryos, and thus litter size (Christenson and Leymaster, 2000).

Among many genes potentially related to reproduction, the most common association with litter size is represented by a gene located on porcine chromosome 8 – osteopontin (*OPN*), commonly referred to as secreted phosphoprotein 1 (*SPP1*) and formerly known as bone sialoprotein 1 (early T-lymphocyte activation 1). OPN/SPP1 is an extracellular matrix (ECM) protein and integrin ligand that is abundantly expressed within the conceptus-maternal environment of pregnancy in numerous species (White et al., 2005).

Expression of this gene in the reproductive tract of various mammalian species has been indicated in several experiments. Monaco et al. (2008) reported that *OPN* gene expression was found in mature and immature oocytes and follicular cells (cumulus cells). Puberty, however, significantly reduces the expression of osteopontin in these cells. The presence of *OPN* mRNA in oocytes and cumulus cells and a greater abundance of mRNA before puberty suggests a significant function of this protein in the maturation of oocytes. However, White et al. (2005) suggest that OPN is a critical component of pregnancy and a significant increase in its expression was observed in the uterus of pigs, sheep, goats, rabbits, mice and humans. Porcine trophectoderm and luminal epithelium cells show evidence of integrin receptor activation and cytoskeletal reorganization in response to OPN binding *in vitro*. Johnson et al. (2003) also suggest that the expression of osteopontin in the uterus was found during the pre-implantation period in humans, pigs and rabbits, which means that OPN is an important protein of epithelium involved in embryo implantation in many mammalian species with different types of implantation and placenta formation. Recent studies on humans and sheep indicate that OPN has a high degree of expression in the glandular epithelium during luteal phase in the endometrium and this expression is regulated by progesterone (Johnson et al., 2000). The importance of this protein was also confirmed during *in vitro* fertilization, both in cattle and in pigs. OPN plays an important role in the regulation of early development of embryos. This mechanism is not clear but it causes a reduction of apoptosis. Finally, localization of the *OPN* gene within the 95% confidence interval of putative quantitative trait loci (QTL) for litter size and prenatal survival (correlated to birth weight) suggests a role of this gene in porcine reproduction (White et al., 2005).

An understanding of the molecular aspects of porcine reproduction is of biological and economic importance. The aim of the study was to find mutations in the regu-

latory and coding regions of the *OPN* gene affecting the expression level of the gene in the ovaries, uterus and oviduct of sows. The evaluation of varying transcription levels of the *OPN* gene undergoing expression in the reproductive tract of sows and their tissue specificity may contribute to the development of knowledge about the genetic background of variability of the reproductive traits in sows. It will also allow selection of mutations associated with the level of the studied traits.

Material and methods

Animals

The material consisted of 71 sows – F_1 hybrids of Polish Landrace and Polish Large White. Animals of the same age were kept under identical environmental conditions and received the same feed. Following the pregnancy period, sows were slaughtered at 10 days after weaning. The tissue samples (pieces of ovary, oviduct, uterine body and uterine horn) were taken at slaughter for the analysis of gene expression and stored in liquid nitrogen. At the same time blood from all animals was collected for DNA extraction. Shortly after slaughter both ovaries from each sow were pictured in four different positions. Pictures were used to define the phase of estrous cycle of sows at slaughter. The data on the anatomy of the reproductive tract were also collected: i.e. the mass of the uterine broad ligament (MUBL), uterine weight without ligaments (UWWL), cervical length (CL), length of the uterine horn (LUH), uterine capacity (UC), length of right and left oviduct (LRO and LLO), left and right ovary weight (LOW and ROL) and the total weight of the ovaries (TWO). The performance data such as: age of the first and second estrus (AFE and ASE), date and age of mating (DM and AM), body weight at mating (BWM), farrowing date (FD), length of pregnancy (LP), number of piglets born alive (NPBA), number of still-born piglets (NSBP), litter weight at birth (LWB), number of piglets at weaning (NPW) and the number of active nipples (NAN) were collected.

Gene polymorphism examination

In the first step extraction and purification of DNA from collected blood samples was performed by phenol-chloroform method. In introns 6 of the *OPN* gene, mutation insertion/deletion type called OPNe6-Knoll was identified. The primers were designed *de novo* on the basis of the polymorphism published by Knoll et al. (1999). The use of these primers allowed amplification of two different fragments consisting of 498 (allele A) or 193 (allele B) base pairs. In the next step new polymorphic sites within the *OPN* gene were sought using the methods: PCR-SSCP (Single Strand Conformation Polymorphism), HRM (High Resolution Melting), sequencing and restriction analysis. In order to search for new polymorphisms, using the sequences of the pig *OPN* gene available in GenBank, several pairs of primers were designed. All primers used for the analysis of the polymorphisms were designed using the Primer3 and PrimerPremier software and verified by widely available tools: Oligo Analyzer 3.1 and Primer-BLAST. The length of all produced

amplicons varied between 100 to 300 base pair fragments. Annealing temperature of the PCR programs for each fragment was experimentally optimized in gradient temperature by MJ Research Tetrad PTC-225 Thermo Cycler. The amplified fragments were visualized on agarose gel using the QIAxcel system (Qiagen). The SSCP analysis of the selected samples was performed. Amplified fragments were checked on the agarose gel and then denatured and separated on 8% polyacrylamide gel in a gradient of temperatures (from 30 to 8°C). The gel was visualized by using a Silver Stain Kit according to the manufacturer's instructions. The fragments characterized by a different amplicon profile were selected. This implies the potential occurrence of polymorphism in various fragments of genes. Both strands of each DNA fragment for four different samples were sequenced to identify the nature of the existing mutation. Another genotyping method used in this study was HRM, a genotyping procedure for identifying mutations. The HRM analyses were performed in duplicate and confirmed the existence of mutations in the studied fragments, which allowed the genotyping without the need of selecting restriction enzymes. Analysis of the data was performed using LightCycler® 480 Multiple Plate Analysis Software.

Gene expression examination

In the next step the frozen tissues (piece of ovary, oviduct, uterine body and uterine horn) were homogenized. RNA isolation was performed with Trizol according to manufacturer's instruction, using the method of Chomczynski (1993). The same samples were also verified by the Bioanalyzer (Perlan). The residue of genomic DNA was removed by digesting the sample with DNase (RNA free) and the RNA was then prescribed for complementary DNA (cDNA). Gene expression at the level of mRNA was studied using real-time PCR. The analysis was performed in several stages. Primers were designed to include a sequence of adjacent exons. The annealing temperatures were chosen experimentally. The analysis was conducted with a Light Cycler 480 (Roche). Expression analysis of *OPN* mRNA for each individual and tissue (ovary, oviduct, uterine body and horn) was performed in duplicate. Real-time PCR analysis was performed to determine the expression dynamics of the selected gene mRNA in the given tissues of the reproductive system. Analyses were performed based on SYBR Green fluorescent dye format. Simultaneously with the analysis of the tested gene, reference genes (also in duplicate) were analysed. The results were normalized in relation to primary metabolism genes: beta-actin (β-actin), a cytoskeletal element and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). Selected reference genes were undergoing stable expression in the reproductive tract. The relative gene expression was determined by the double-delta method. A comparison was made for relative expression in different tissues and different mutations.

Statistical methods

In the first stage the ALLELE procedure of SAS/STAT (2010) was used to estimate the frequency of alleles and genotypes in different loci, heterozygosity and gene diversity, genetic equilibrium on the basis of Hardy-Weinberg law (chi^2 test) and polymorphism information content (PIC) value. In the second stage the analysis

of variance (GLM procedure of SAS/STAT) of the level of expression (mRNA) of the OPN gene was performed. The values of the level of expression of the *OPN* gene were log-trasformed to the base 10 in order to normalize distribution. The first model was fitted for phase of the estrous cycle, individual tissues (ovary, oviduct, uterine body and horn) and interaction between phase and tissue. The second model was fitted for *OPN* gene mutations, individual tissue and interactions of tissue by gene mutation. The estrous cycle phase was not included in the analysis as it was insignificant in the first model. The significance of differences between levels of evaluated traits was estimated by Duncan's test. There was a linear relationship between transcript level and phenotypes, which justified the use of linear regression analysis. In the next stage, regression of the levels of expression (mRNA) of the *OPN* gene in individual tissues and the traits of reproductive performance and the reproductive system traits of sows was also conducted.

Results

Among the 71 individuals two groups of sows, 46 in luteal and 25 in follicular phase were found. Within the mutation OPNe6 Knoll in intron 6 three different forms of genotype (AA, AB, BB) were found. In the examined material the frequency of allele A (498 base pairs) and allele B (193 base pairs) was 0.39 and 0.61, respectively (Table 3).

Table 1. Allele-specific primers to amplify the OPNp3–4 fragment in the promoter of the osteopontin gene

Position	Primer sequence
–606A	ATGGATTTGAACGTGACCAA
–606G	GTGGATTTGAACGTGACCAA
–617A	TTATTAAATCGGATTTACCATTGA
–617G	CTATTAAATCGGATTTACCATTGTA

Table 2. The number and frequency of different genotype groups in the studied loci

Locus	Genotype	Number of animals in the genotype groups	Frequency of genotypes	
		observed	observed	expected
OPNe6-Knoll	AA	10	0.1408	0.1555
	AB	36	0.5070	0.4776
	BB	25	0.3521	0.3667
OPNp3-4	AA	9	0.1268	0.2031
	AG	46	0.6479	0.4951
	GG	16	0.2254	0.3017
OPNe6-1	AA	8	0.1127	0.0607
	AC	19	0.2676	0.3714
	CC	44	0.6197	0.5677

Table 3. Allele frequencies, heterozygosity and gene diversity, PIC and chi^2 value in the studied loci

Locus	Allele	Frequencies	PIC	Heterozygosity	Allelic diversity	Chi^2	Pr>Chi^2
OPNe6-KnoII	A	0.3944	0.3636	0.5070	0.4777	0.2682	0.6045
	B	0.6056					
OPNp3-4	A	0.4507	0.3726	0.6479	0.4951	6.7569	0.0093
	G	0.5493					
OPNe6-1	A	0.2465	0.3025	0.2676	0.3715	5.5494	0.0185
	C	0.7535					

Searching for new polymorphisms within the *OPN* gene

Within the *OPN* gene, the following mutations were identified in the promoter of the gene: two point mutations SNP type (Single Nucleotide Polymorphism) and one microsatellite sequence. One of the amplified fragments with a length of 220 bp (named OPNp1-2) was characterized by the presence of microsatellite sequences (TG)n, which was also confirmed by results of SSCP analysis. This fragment was found to be unsuitable for further analysis. Another fragment, with a size of 274 bp (named OPNp3-4), and showing a different pattern of conformers, was sequenced using an ABI PRISM 3130 sequencer. The resulting sequence was characterized by the presence of a few specific sites: CACCTCC – type II collagen silence sequence at position –682 from the transcription initiation site, TGTCCT – a site responsive to glucocorticoids in position –658 and a "CAAT box" at position –592. Results of sequencing have also enabled transition A→G at positions –617 and –606 to be detected within the tested fragment of gene. Restriction analysis using Webcutter software was performed, but none of the known restrictive enzymes recognized the newly identified SNPs. The sequence analysis indicates the possibility of using two characteristic primer pairs for specific allele, determining the presence of either nucleotide A or G (Table 1). This polymorphism was studied in 71 sows with the frequency of 0.45 for allele A and 0.55 for allele G (Table 3). In the exon 6 of the OPN gene, within the 110 base pairs fragment two synonymous mutations were identified: the exchange of nucleotide A to C occurred in position –462 and the exchange of nucleotide C to G occurred in position –477. This mutation was called OPNe6-1 and was confirmed by the HRM analysis. Two types of alleles: A and C were found with frequency of alleles 0.25 and 0.75, respectively (Table 3). Within the *OPN* gene a mutation in exon 7, named OPNe7-1 was identified. The OPNe7-1 mutation consists of an exchange of nucleotide A to G in position –603 within the 149 base pair fragment. The HRM analysis identified three genotypes, with the predominant frequency of homozygote GG. For 71 examined sows, 65 showed this genotype. There were only two individuals of the second type homozygote – AA and four heterozygous AG animals. This fragment was found to be unsuitable for further analysis.

Results of *OPN* gene frequencies

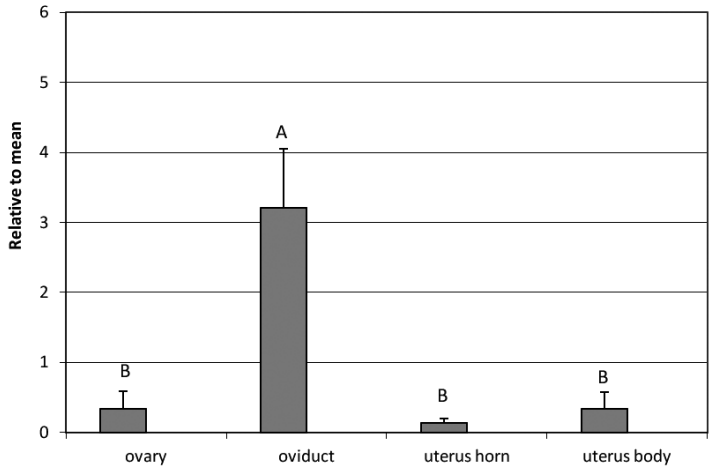
The frequencies of alleles and genotypes in all mutation sites were presented in Tables 2 and 3. The observed heterozygosity in this population was 0.5070 for

OPNe6-Knol and 0.6479 for OPNp3-4, which demonstrates that the herd was characterized by high variability and 0.2676 for OPNe6-1, which shows low variability in this locus. The expected heterozygosity (allele diversity) determines the probability with which two randomly selected alleles in the population are different and it was 0.4777, 0.4951 and 0.3715 for loci OPNe6-Knol, OPNp3-4 and OPNe6-1, respectively. PIC factor can determine the probability of the origin of the parent allele and amounts to 0.3636, 0.3726 and 0.3025, respectively to the polymorphic site. The comparison between the number of observed genotypes and the theoretical number of genotypes showed loss of genetic equilibrium in the investigated population. Chi^2 values allow for assessment of the genetic equilibrium in the population. Thus, the loci OPNp3-4 and OPNe6-1 were in genetic disequilibrium while locus OPNe6-Knoll showed genetic equilibrium.

Results of *OPN* gene expression examination

OPN gene expression was analysed at the mRNA level. RNA of good quality, suitable for expression analysis of samples was isolated from ovaries of 58 individuals and from oviducts of 53 individuals and also from samples of the uterus (horns and body) of 26 individuals. A significantly ($P \leq 0.01$) higher level of osteopontin mRNA was found in the oviduct in comparison to the other examined tissues (Fig. 1). The statistical analysis of relationship between the level of *OPN* mRNA in different tissues (ovary, oviduct, uterine horn and body) and described polymorphisms (OPNe6-Knoll,OPNp3-4, OPNe6-1) produced the following results: There was a significant difference between the expression of osteopontin in the uterine horn, uterine body and polymorphism located in the promoter (OPNp3-4) (Table 4). The AA homozygote showed higher levels of expression of osteopontin in the uterine horn in relation to the heterozygous AG and homozygous GG. In the uterine body, the situation was the opposite – GG homozygotes showed higher expression than the other genotypes. There were also significant differences between the level of expression of these same genotypes in different tissues. For example AA genotype in exon 6 (OPNe6-1), the AG in the promoter (OPNp3-4) and the AB and BB in intron 6 (OPNe6-Knoll) were characterized by the highest level of expression in the oviduct. Only animals with the GG genotype of polymorphism within the promoter have the highest level of expression in the body of the uterus. All these differences are statistically significant at $P<0.05$ (Table 5).

The relationships between levels of expression of the *OPN* gene in examined reproductive tissues and investigated characteristics of the reproductive system and traits of reproductive performance are presented in Table 6. The table includes only those traits for which the mRNA level showed a significant correlation. The expression level of the *OPN* gene in the oviduct was significantly ($P \leq 0.01$) correlated with age of mating and number of active nipples. It was also significantly ($P<0.05$) associated with litter weight at birth, age at slaughter and cervical length. The expression level of the *OPN* gene in the uterine body was significantly correlated at $P \leq 0.01$ with cervical length, litter weight at birth and age at slaughter and significantly correlated at $P<0.05$ with age of mating and with body weight at mating.



A, B – different letters show significantly different expression levels (P≤0.01)

Figure 1. Expression level of osteopontin in examined tissues

Table 4. The relationship between the *OPN* expression level (mRNA) in uterine horn and body and described OPNp 3–4 polymorphism

Expression level in selected tissues			
OPN genotype		uterine horn LSM /Se	uterine body LSM /Se
OPNp3–4	AA	–0.21/0.73 a*	–2.39/0.73 b
	AG	–2.16/0.35 b	–1.63/0.35 b
	GG	–2.34/0.65 b	–0.05/0.65 a

LSM – Least Square Means; Se – standard error.

Levels of significance: a, b – LSM values in the columns with different letters differ significantly at P<0.05;

* Negative values indicate lower expression relative to reference genes.

Table 5. The relationship between the expression level (mRNA) of described genotype of *OPN* and various tissues of sow's reproductive system

Expression level in selected tissues					
OPN genotype		ovary LSM /Se	oviduct LSM /Se	uterine horn LSM /Se	uterine body LSM /Se
OPNe6–1	AA	–1.80/0.57 b*	–0.04/057 a	–2.24/0.75 b	–0.30/0.75
	AC	–1.15/0.40	–1.05/0.47	–1.86/0/75	–1.71/0.75
	CC	–1.46/0.25	–0.69/0.25 a	–1.83/0.35 b	–1.64/0.35 b
OPNp3-4	AA	–1.27/0.55	–0.92/0.55	–0.21/0.73 a	–2.39/0.73 b
	AG	–1.42/0.23 b	–0.72/0.25 a	–2.16/0.35 b	–1.63/0.35 b
	GG	–1.53/0.44	–0.35/0.46 a	–2.34/0.65 b	–0.05/0.65 a
OPNe6-Knoll	AA	–1.45/0.53	–0.69/0.68	–0.67/1.07	–2.10/1.07
	AB	–1.44/0.27 b	–0.61/0.28 a	–1.86/0.38 b	–1.59/0.38 b
	BB	–1.37/0.35	–0.76/0.35 a	–2.27/0.53 b	–0.99/0.53

LSM – Least Square Means; Se – standard error.

Levels of significance: a, b – LSM values in the rows (within the individual polymorphic site) with different letters differ significantly at P<0.05.

* Negative values indicate lower expression relative to reference genes.

Table 6. Regression coefficient between OPN expression level in selected tissues and studied reproductive system and traits of reproductive performance

Traits	Expression level in selected tissues			
	OPN oviduct		OPN uterine body	
	B	Se	B	Se
AM	–4.9043**	1.6840	–5.0158*	1.4040
CL	0.4513*	0.2149	0.5345**	0.1911
LWB	–0.6520*	0.2886	–0.9615**	0.2369
NAN	–0.2954**	0.1098		
AS	–4.5863*	1.8233	–5.4503**	1.5421
BWM	–	–	–1.5506*	0.7403

B – Regression coefficient, Se – standard error.

Abbreviations used: AM – age of mating, CL – cervical length, LWB – litter weight at birth, NAN – number of active nipples, AS – age at slaughter, BWM – body weight at mating.

*, ** – levels of significance,*P<0.05, **P≤0.01.

Discussion

Within the osteopontin gene several polymorphisms were found. Some of them may be useful for further studies, such as investigating whether a mutation within the promoter may be related to the regulation of transcription or whether a mutation within exon 6 may also be associated with the functioning of the gene, because the mutation affects encoding fragments. Translation of osteopontin sequence of exon 6 revealed that the described mutation is positioned in a highly conservative RGD cell attachment sequence (Garlow et al., 2002). RGD sequence is recognized by integrins and constitutes a major recognition system for cell adhesion (Ruoslahti, 1996) which is essential in the process of implantation. The moment of adhesion of the embryo to the apical surface of trophoectoderm luminal epithelium is extremely important in this process. It is considered that the main role in this process is played by adhesion molecules, mostly integrin of av family anchoring to matrix proteins like osteopontin (Aplin and Kimber, 2004).

The described deletion of the gene fragment in intron 6 (OPNe6-Knoll), already mentioned by other authors (Hao et al., 2008), showed a relationship with traits such as litter size. Interestingly, however, variation in a microsatellite repeat of the *OPN* gene was previously shown to be associated with an increase in litter size in a Meishan × Large White cross (Van der Steen et al., 1997). This marker lies within the 95% confidence intervals for the litter-size and embryo-survival QTL. More recently, Korwin-Kossakowska et al. (2002) reported associations between the presence of a SINE (Short Interspersed Nucleotide Element) in the *OPN* gene and litter size of the second and subsequent parities for 519 sows from a commercial Polish line.

At the same time the gene expression in ovary, uterus and oviduct was measured. Previous studies of the *OPN* gene have revealed that it is expressed in a variety of tissues, including the epithelial cells of the endometrial and metrial gland cells of the decidua within the uterus, and the invading trophoblast, during the defined window

of receptivity of the peri-implantation period of pregnancy in several mammalian species (Johnson et al., 1999; King et al., 2003; Nomura et al., 1988). In our study, gene expression in the reproductive tract of examined sows was very diverse, considering its low level in the uterus and high level within the oviduct. Based on the novel polymorphisms a significant correlation between the *OPN* genotype and *OPN* expression (mRNA) level in ovary, oviduct, the uterine body and horn was observed.

The oviduct and uterus in mammals are the venue of important actions leading to the establishment of pregnancy, including final maturation and transport of the gametes, fertilization, embryonic development, transport of the embryo to the uterus, development to the blastocyst stage, elongation and then blastocyst attachment to the uterus. During the estrous cycle, the tract undergoes significant endocrine-induced morphological, biochemical, and physiological changes which establish an essential microenvironment within the oviduct and uterus (Goluch et al., 2009). In the current study, the level of the *OPN* expression in both the oviduct and the uterus depends on various factors listed below such as mating age, length of the cervix, litter weight at birth, the sow weight and age at slaughter. Interestingly, the *OPN* expression in the uterus (both horn and the body) was associated with polymorphisms in promoter and also had an impact on litter weight at birth (unpublished data).

A comprehensive analysis of temporal and spatial patterns of *OPN* mRNA and protein expression within the uteri of multiple species suggests that *OPN* has diverse biological functions in the uterus (Johnson et al., 2003) throughout the estrous cycle and during the pre-implantation period in early pregnancy. Also Garlow et al. (2002) previously noted an association between the initiation of *OPN* mRNA and protein expression in the uterine luminal epithelium, coinciding with conceptus elongation, pregnancy recognition, and early attachment to the uterine wall for implantation and suggested that a paracrine factor from the conceptus may be responsible for this expression event.

The results presented here may serve as a starting point for further studies covering problems related to the expression of the *OPN* gene within the period of early pregnancy, as well as from 14 to 16 days of pregnancy when embryo implantation occurs.

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Polimorfizm w genie osteopontyny i poziom jej ekspresji w układzie rozrodczym loch

STRESZCZENIE

Celem badań było znalezienie polimorfizmu genu osteopontyny (*OPN*) jako potencjalnego miejsca mutacji mającej związek z ekspresją tego genu na poziomie mRNA w jajnikach, macicy i jajowodzie. Materiał stanowiło 71 loch ras wbp × pbz. W pierwszym etapie badań opracowano kilka polimorfizmów w obrębie promotora, intronu 6 oraz eksonów 6 i 7 genu osteopontyny. Oszacowano frekwencję genotypów i alleli, heterozygotyczność oczekiwaną i spodziewaną,, współczynniki PIC oraz chi^2. Stwierdzono, że w obrębie dwóch loci badana populacja nie znajdowała się w stanie równowagi genetycznej, natomiast w trzecim locus, tj. OPNe6-Knoll taka równowaga została zachowana. Wykonano również analizę real time PCR w celu określenia poziomu ekspresji w badanych tkankach w odniesieniu do genów ulegających stabilnej ekspresji w układzie rozrodczym. Dokonano porównania poziomu relatywnej ekspresji w różnych tkankach i w odniesieniu do różnych mutacji. Najwyższy poziom ekspresji odnotowano w jajowodzie. Bazując na nowo opracowanych mutacjach zaobserwowano wiele istotnych zależności pomiędzy genotypem *OPN* a poziomem ekspresji w jajniku, jajowodzie, rogach i trzonie macicy. W drugim etapie badań porównywano poziom ekspresji w poszczególnych częściach układu rozrodczego i poziom cech związanych z rozrodem i budową układu rozrodczego. Poziom ekspresji w jajowodzie miał związek z wiekiem krycia, liczbą aktywnych sutków, długością szyjki macicy, masą miotu przy urodzeniu, i masą ciała przy uboju. Natomiast poziom ekspresji w trzonie macicy miał związek z długością szyjki macicy, masą miotu przy urodzeniu i wiekiem uboju, a także wiekiem krycia i masą ciała przy kryciu.