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Expression changes in fatty acid metabolic process-related genes in porcine oocytes during *in vitro* maturation

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Abstract

Mammalian oocytes undergo compound processes of nuclear and cytoplasmic maturation that allow them to reach MII stage. Only fully mature, oocyte can be successfully fertilized by a single spermatozoon. Fatty acids, apart from their role in cellular metabolism, inflammation and tissue development, have positive and detrimental effects on oocyte maturation, fertilization, blastocyst cleavage rate and embryo development in mammals. Using microarrays, we have analyzed the expression changes in fatty acids--related genes during in vitro maturation of porcine oocytes.

The oocytes were recovered from ovaries of 45 pubertal crossbred Landrace gilts and subsequently subjected to BCB test. For further analyses, only granulosa cell-free BCB+ oocytes were used and divided into two groups. The first one, described as "before IVM", was directly exposed to molecular assays, the second one, described as "after IVM", was first in vitro matured and then subjected to a second BCB test. Oocytes, if classified as BCB+, were then passed to corresponding molecular analyses.

We found significant down-regulation of genes involved in fatty acid metabolic process, such as: ACSL6, EPHX2, FADS2, PTGES, TPI1, TBXAS1, NDUFAB1, MIF, ACADSB and DECR1 in porcine oocytes analyzed after IVM, in comparison to those analyzed before IVM.

In conclusion, apart from poor data available concerning analyzed genes in relation to reproductive events, significant changes in their expression point to their potential role as an oocyte developmental competence markers in pigs. Introducing molecular diagnostics of oocytes could be the prospective tool for selection of best gametes, leading to improved outcomes of in vitro fertilization.

Running title: Fatty acids changes in porcine oocytes matured in vitro

Keywords: porcine oocytes, fatty acids, *in vitro* maturation (IVM), microarrays

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Introduction

Ovarian follicles of mammals are composed of cumulus-oocyte complexes (COCs) and surrounding somatic granulosa and theca cells. The process of follicle growth, called folliculogenesis, includes numerous stages of primary, secondary, antral and fully matured Graafian follicle [1]. Both, *in vivo* and *in vitro*, folliculogenesis is accompanied by the process of oogenesis- the maturation of the oocyte. The latter is very complex and involves stages of germinal vesicle (GV), germinal vesicle breakdown (GVBD), metaphase I (MI), ending with the mature oocyte at metaphase II (MII) [2]. During these stages an oocyte grows and undergoes several morphological and biochemical modifications. The first ones include cumulus cells' (CCs) expansion and structural and ultrastructural changes in the oocyte's organelles [3]. The second group includes nuclear maturation, leading to achievement of an appropriate chromosome configuration by the oocyte, and cytoplasmic, which is accompanied by the accumulation of mRNA and proteins necessary for successful fertilization and further early embryo growth [4]. Thus, all mentioned steps are crucial for the oocyte to acquire developmental competency, manifested by the fertilisation ability and proper embryo growth [5]. Therefore, intracellular modifications, taking place during maturation process, may be the marker of oocyte's quality, as well as the developmental potential of *in vitro* matured gametes. Consequently, molecular alterations underlying this process are crucial to be defined.

Thus, we analysed changes in genes expression profile in immature and mature porcine oocytes using Affymetrix microarray assays [6,7]. We selected genes belonging to fatty acid metabolic process ontology group. Our selection was caused by the fact that apart from their role in cellular metabolism, inflammation and tissue development [8] fatty acids have been involved in cell-cell interactions, membrane transport and signaling events, which are crucial in reproductive events [9]. It was shown, that fatty acids can have detrimental effects on oocyte maturation, fertilization, blastocyst cleavage rate and embryo development in mammals [10].

All this together points to a possibility that, also in pig oocytes, fatty acid administration can influence the outcome of maturation. We focused on spontaneous changes of fatty acids during porcine oocyte *in vitro* maturation, suggesting potential gene markers of their developmental compatibility.

Material and methods

Experimental design

Oocytes were collected and, after double Brilliant Cresyl Blue (BCB) test, divided into two groups. The first group ("before IVM") included oocytes graded as BCB-positive (BCB^+) and directly exposed to microarray assay and RT-qPCR. The second group ("after IVM") included BCB^+ oocytes which were then matured *in vitro* and, if classified as BCB^+ in second BCB test, passed to molecular analyses.

Animals

A total of 45 pubertal crossbred Landrace gilts bred on a commercial local farm were used in this study. They had a mean age of 155 days (range 140 – 170 days) and weight of 100 kg (95-120 kg). All animals were bred under the same conditions and fed the same forage (depending on age and reproductive status). All experiments were approved by the Local Ethic Committee.

Collection of porcine ovaries and cumulus-oocyte-complexes (COCs)

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 40 min at 38°C in 0.9% NaCl. To provide optimal conditions for subsequent oocyte maturation and *in vitro*, the ovaries of each animal were placed in a 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. Single large follicles (>5mm) were opened by puncturing with a 5ml syringe and 20-G needle in a sterile Petri dish, COCs were recovered. The COCs were washed three times in modified PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamicin, and 0.5 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). The COCs were selected under an inverted microscope- Zeiss, Axiovert 35 (Lübeck, Germany), counted, and morphologically evaluated. Only COCs of grade I, possessing homogeneous cytoplasm and uniform, compact cumulus cells, were considered for further use, resulting in a total of 300 grade I oocytes (3 x n=50 "before IVM" group, 3 x n=50 "after IVM" group).

Assessment of oocyte developmental competence by BCB test

Brilliant Cresyl Blue (BCB) test was used for assessment of porcine oocytes' quality and maturity [11]. The glucose-6-phosphate (G6PDH) enzyme converts BCB stain from blue to colorless. In oocytes that completed the growth, activity of the enzyme decreases, the stain cannot be reduced, resulting in blue oocytes (BCB^+). To perform the BCB staining test, oocytes were washed twice in modified Dulbecco's Phosphate Buffered Saline (DPBS), commercially supplemented with 0.9 mM calcium, 0.49 mM magnesium, 0.33 mM pyruvate, and 5.5 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), and additionally with 50 IU/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0.4% Bovine Serum Albumin (BSA) [w/v] (Sigma-Aldrich, St. Louis, MO, USA). They were then treated with 13 μM BCB (Sigma-Aldrich, St. Louis, MO) diluted in DPBS at 38.5°C, 5% CO_2 for 90 min. After treatment, the oocytes were transferred to DPBS and washed twice. During washing, the oocytes were examined under

an inverted microscope and classified as stained blue (BCB^+), or colorless (BCB^-). Only the granulosa cell-free BCB^+ oocytes were used for subsequent molecular analyses ("before IVM" group), or IVM, followed by second BCB test and molecular analyses ("after IVM" group).

In vitro maturation of porcine cumulus-oocyte-complexes (COCs)

After the first BCB test, the BCB^+ COCs were subjected to IVM. The COCs were cultured in Nunclon™Δ 4-well dishes (Thermo Fisher Scientific, Waltham, MA, USA) in 500 µl standard porcine IVM culture medium: TCM-199 (tissue culture medium) with Earle's salts and *L*-glutamine (Gibco BRL Life Technologies, Grand Island, NY, USA), supplemented with 2.2 mg/ml sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 10 mg/ml BSA (Bovine Serum Albumin) (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml cysteine (Sigma-Aldrich, St. Louis, MO, USA), 10% (v/v) filtered porcine follicular fluid, and gonadotropin supplements at final concentrations of 2.5 IU/ml hCG (human Chorionic Gonadotropin) (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/ml eCG (equine Chorionic Gonadotropin) (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38° C under 5% CO_2 in air for 22h, and then for additional 22h in medium without hormones. After cultivation, the second BCB staining test was performed, and BCB^+ oocytes were used for further molecular analyses.

RNA extraction from porcine oocytes

Total RNA was extracted from samples using TRI Reagent (Sigma, St Louis, MO, USA) and RNeasy MinElute cleanup Kit (Qiagen, Hilden, Germany). The amount of total mRNA was determined using optical density at 260 nm, and the RNA purity was estimated using the 260/280 nm absorption ratio (higher than 1.8) (NanoDrop spectrophotometer, Thermo Scientific, ALAB, Poland). The RNA integrity and quality were checked on a Bioanalyzer 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA). The resulting RNA integrity numbers (RINs) were between 8.5 and 10 with an average of 9.2. The RNA in each sample was diluted to a concentration of 100 ng/µl with an OD260/OD280 ratio of 1.8/2.0. From each RNA sample, 500 ng of RNA were taken. The remaining amount of isolated RNA was used for RT-qPCR study.

Microarray expression analysis and statistics

Experiments were performed in three replicates. Total RNA (100 ng) from each pooled sample was subjected to two round sense cDNA amplification (Ambion® WT Expression Kit). The obtained cDNA was used for biotin labeling and fragmentation by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labeled fragments of cDNA (5.5 µg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (48°C/20 h). Then, microarrays were washed and stained according to the technical protocol, using Affymetrix GeneAtlas Fluidics Station. The array strips were scanned, employing Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed, using Affymetrix GeneAtlasTM Operating Software. Quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis software.

All analyses were performed using BioConductor software, based on the statistical R programming language. For background correction, normalization and summation of raw data, the Robust Multiarray Averaging (RMA) algorithm, implemented in "affy" package of BioConductor, was applied. Biological annotation was taken from BioConductor "oligo" package, where annotated data frame object was merged with normalized data set, resulting in a complete gene data table. Statistical significance of analyzed genes was performed by moderated t-statistics from the empirical Bayes method. Obtained p value was corrected, for multiple comparisons, using the Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on p value beneath 0.05 and expression fold higher than |2|.

Functional annotation clustering of differentially expressed genes was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery). Gene symbols for up- or down-regulated genes, from each of the compared groups, were loaded to DAVID by "RDAVIDWebService" BioConductor package. In this analysis we focused on one GO term group described as "fatty acid metabolic process", that was separated from other GO groups and subjected to hierarchical clusterization algorithm and presented as a heat map graph. "fatty acid metabolic process" GO term (GO:0022610) belongs to Biological Process GO domain.

Interactions between differentially expressed genes/proteins belonging to "fatty acid metabolic process" ontology group were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes). List of gene names were used as query for interaction prediction. Searching criteria were based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression and experimentally observed interactions. The results of such analysis generated gene/protein interaction network where the intensity of the edges reflects the strength of interaction score. Besides interaction prediction, STRING also allowed us to perform functional enrichments of GO terms based on previously uploaded gene set from "fatty acid metabolic process" GO BP term.

Finally, we checked if the genes that belongs to "fatty acid metabolic process" ontology group are also part of any pathway defined by pathway database. In that purpose we used Consensus Path Data Base, which integrates interaction networks in Homo sapiens including binary and complex protein-protein, genetic, metabolic, signaling, gene regulatory and drug-target interactions, as well as biochemical pathways. In our analysis we looked for pathway that contains at least 5 of studied genes. We search through pathways from Wikipathways, Netpath, Pharmgkb, Kegg, Humaneye, Ehmn, Inoh, Smpdb, Biocarta, Reactome, Signalink and Pid databases. The enriched pathways were visualized in Cytoscape 3.5.0 software.

Ethical approval:

The research related to animal use has been complied with all the relevant national regulations and institutional policies for the care and use of animals. Bioethical Committee approval no. 32/2012.

Results

Whole transcriptome profiling by Affymetrix microarray allowed us to analyze the gene expression changes in freshly isolated oocytes, before *in vitro* procedure ("before IVM"), in relation to after *in vitro* maturation ("after IVM"). By Affymetrix® Porcine Gene 1.1 ST Array, we have examined expression of 12258 porcine transcripts. Genes with fold change higher than |2| and with corrected p value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 419 different transcripts. Subsequently, the genes were used for identification of significantly enriched GO BP terms.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of the genes belonging to "fatty acid metabolic process" gene ontology Biological Process term (GO BP). We found that 10 genes from "fatty acid metabolic process" GO BP term were significantly represented in down-regulated gene set. This set of genes was subjected to hierarchical clusterization procedure and presented as heat map (**Fig. 1**).

Set of the differentially expressed genes belonging to "fatty acid metabolic process" GO BP term with their official gene symbols, fold changes in expression, corrected p values and Entrez gene IDs were shown (**Tab. 1**).

STRING-generated interaction network was created with differentially expressed genes belonging to the "fatty acid metabolic process" ontology group. The intensity of the edges reflects the strength of interaction score (**Fig. 2**).

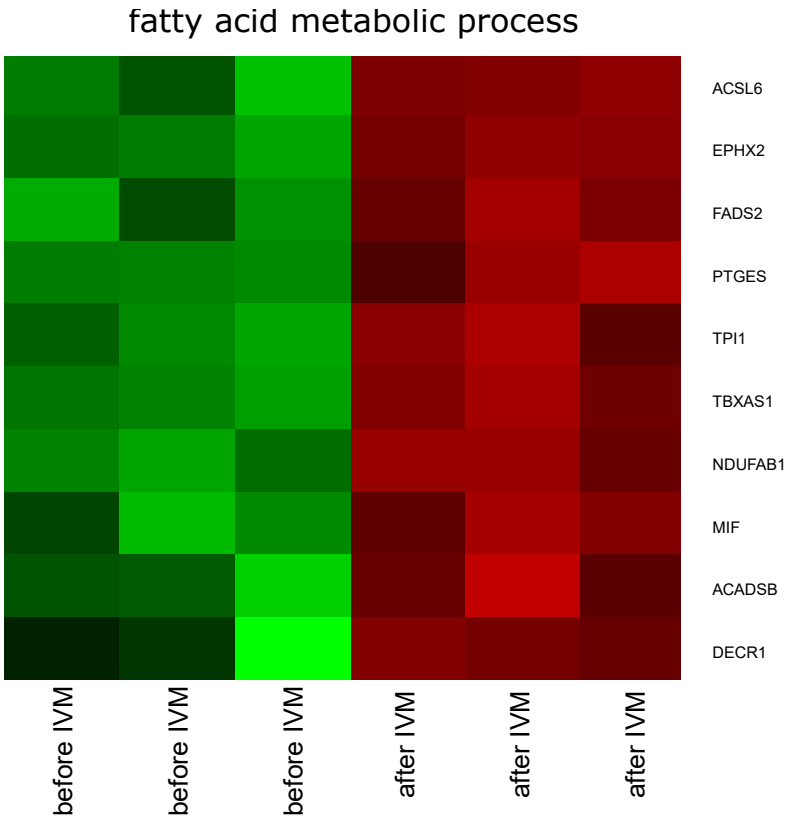

Figure 1 Heat map representation of differentially expressed genes belonging to the "fatty acid metabolic process" GO BP term. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower expression). Log2 signal intensity values for any single gene were resized to Row Z-Score scale (from -2, the lowest expression to +2, the highest expression for single gene)

Table 1 Gene symbols, fold changes in expression, corrected p values and Entrez gene IDs of studied genes

Gene symbol	Fold change	Corrected p values	Entrez gene ID
EPHX2	0.150135	0.000134	2053
NDUFAB1	0.204763	0.000312	4706
PTGES	0.337362	0.001205	9536
MIF	0.375718	0.002882	4282
FADS2	0.383543	0.00222	9415
TBXAS1	0.387156	0.000479	6916
ACSL6	0.389445	0.001953	23305
DECR1	0.415159	0.042461	1666
ACADSB	0.478811	0.012394	36
TPI1	0.483392	0.002063	7167

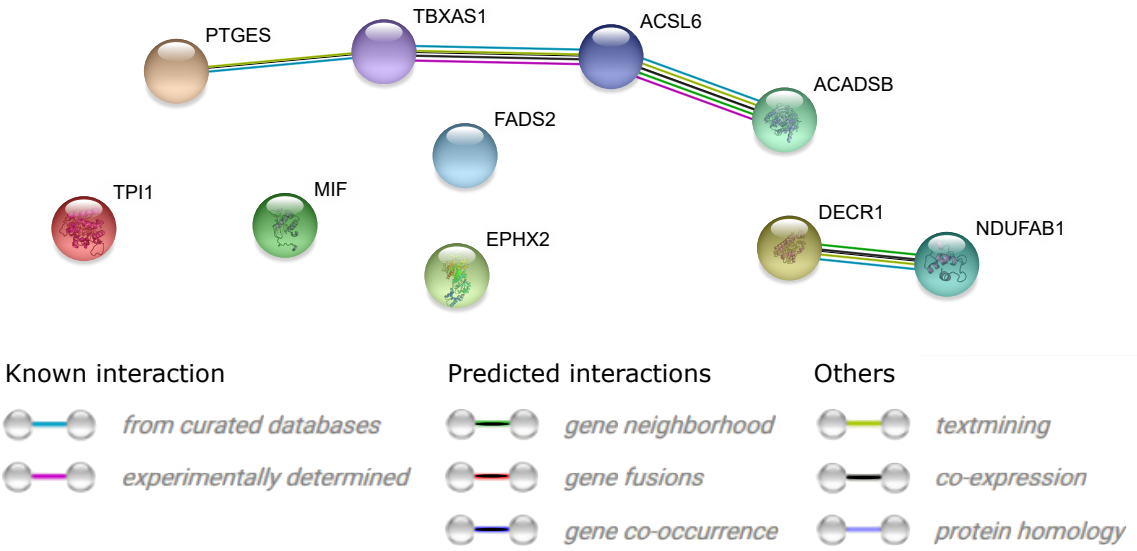

Figure 2 STRING-generated interaction network among differentially expressed genes belonging to the "fatty acid metabolic process" GO BP term. The intensity of the edges reflects the strength of interaction score

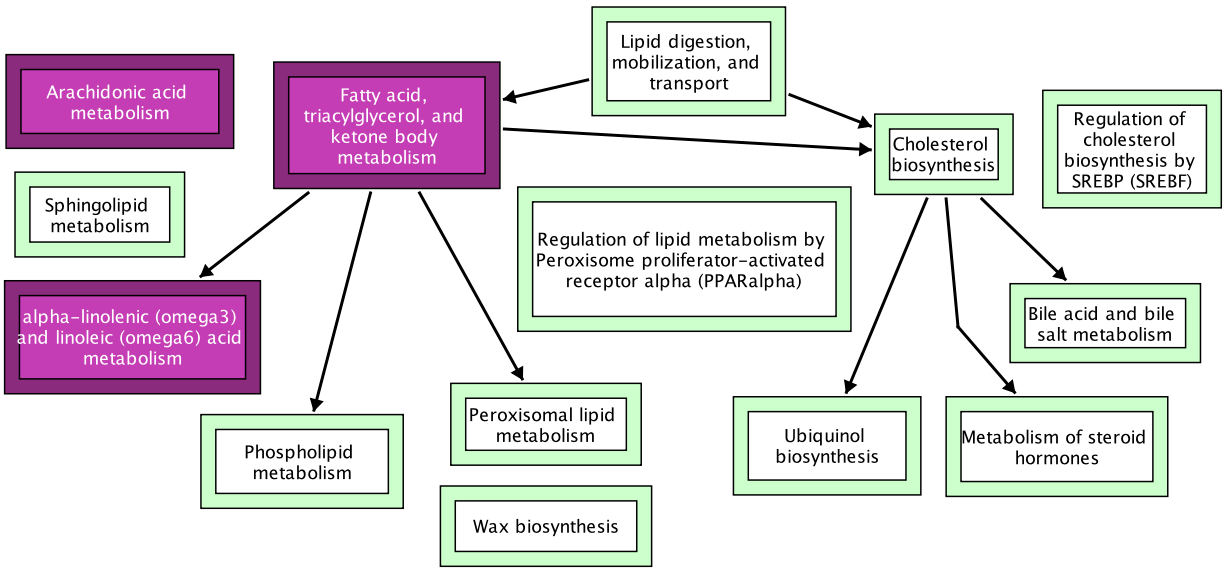

Figure 3 The diagram of "Metabolism of Lipids and proteins" REACTOME pathway. The part of this pathway containing genes from "fatty acid metabolic process" GO BP term are marked purple

Finally, the enrichment analysis of pathways from different databases showed that 5 or more genes show only in "Metabolism of Lipids and proteins" pathway from REACTOME database. The selected genes were FADS2, EPHX2, NDUFAB1, TBXAS1, ACSL6, PTGES. The genes of mentioned pathway were involved in "Fatty acid, triacylglycerol, and ketone body metabolism", "Arachidonic acid metabolism" and "alpha-linolenic (omega 3) and linoleic (omega 6) acid metabolism". The diagram of upregulated pathway was shown in **Figure 3**.

Discussion

Oocyte quality is a factor which has a great influence not only on their maturation capability but also on successful fertilization, blastocyst formation or even early embryo development [5]. Thus, we focused on porcine oocyte *in vitro* maturation process which, even if well known, still needs some improvements to increase blastocyst yield. Since it is well known that oocyte undergoes biochemical alterations during *in vitro* maturation, including transcriptomic profile changes, its mRNA is a great source of information on potential markers of developmental capability. These alterations in gene expression can mirror synthesis of proteins necessary for acquiring full maturity, fertilization ability and proper growth of the early embryo [12] and thus, selection of high quality oocytes can increase prospective IVF outcome.

Nowadays, there is a growing interest in fatty acids influence on reproductive processes. It was already elucidated that elevated levels on non-esterified fatty acids (NEFA) are mirrored in ovarian follicles of cattle and human, having unfavorable effect on oocytes maturation and fertilization capacity [13,14]. Furthermore, blastocysts obtained from oocytes matured with addition of NEFA presented decreased cell counts and lowered quality [15]. Also, somatic cells coexisting in the ovarian follicle, like granulosa and theca cells, were susceptible to NEFA administration, resulting in impaired proliferation rate and process of steroidogenesis [16]. On the contrary, Lee et al. found that treatment with alpha-linoleic acid during oocytes' maturation improved their developmental competence by accelerating nuclear maturation and influencing cytoplasmic maturation [17].

To analyze spontaneous changes of fatty acids administration in porcine oocytes during IVM, we selected several genes belonging to fatty acids associated ontology groups, like: *ACSL6*, *EPHX2*, *FADS2*, *PTGES*, *TPI1*, *TBXAS1*, *NDUFAB1*, *MIF*, *ACADSB* and *DECR1*. All of them presented significant down-regulated expression after standard IVM procedure. Among them three brought our attention.

Prostaglandin E Synthase (PTGES) catalyzes the oxidoreduction of prostaglandin endoperoxide H2 (PGH2) to prostaglandin E2 (PGE2). PTGES was found both on mRNA and protein level during ovarian development in human. Expression was predominantly found in germ cells with expression increasing along with gestation, suggesting PTGES role in germ cells development. This observation confirms PGE2's role in ovarian development, being most likely is responsible for subsequent activation of genes important for oocyte persistence and maturation. Additionally, it can be involved in connections between germ and somatic cells during ovarian follicle formation [18]. Furthermore, the same interactions were observed in the postnatal life in cattle, where prostaglandins are regarded as responsible for cumulus-oocyte complex creation and play critical role during pre-ovulatory COCs expansion and oocyte maturation [19]. The pre-ovulatory surge of gonadotropins increases PTGES level in cumulus cells, and thus leads to PGE2 up-regulation [20,21]. The latter is crucial for release of the oocyte but probably not for follicle rupture, and its luteinization [22]. Changes in PGE2 and progesterone level are hallmarks of final COCs differentiation and influence developmental competence of the oocyte [23].

Another gene the Macrophage Migration Inhibitory Factor (MIF), encodes a protein predominately associated with macrophages. It regulates their function by suppressing anti-inflammatory effects of glucocorticoids at infection sites. However, there are many other properties of MIF already revealed. Among them *MIF* mRNA expression was found in murine ovulated oocytes, zygotes, 2-cell embryos, 8-cell embryos, blastocyst and reproductive organs, like uterus and oviduct. The presence of transcripts of that gene was recorded in all of them, however its highest expression occurred in uterus during pregnancy and embryos, suggesting yet unknown function of MIF in establishing early pregnancy [24]. In human ovary, specifically in follicular fluid, high levels of MIF were also observed. It was attributed to the granulosa cells present, which have been shown to manifest potential for *de novo* MIF synthesis. Interestingly, in bigger follicles, level of MIF was lower, suggesting that during maturation process granulosa cells lose efficiency in MIF production. All this stays in accordance with observation that MIF can contribute to steroidogenesis via glucocorticoid and insulin-like growth factor I actions. It proves that MIF, apart from known function in immunological reactions, also plays a role in oocytes' maturation through process of steroidogenesis mediated by the granulosa cells [25].

Finally, the last gene - Acyl-CoA Synthetase Long Chain Family Member 6 (*ACSL6*) mediates formation of acyl-CoA from fatty acids, ATP and CoA. It is involved in fatty acid metabolism, especially in the brain, and takes part both in fatty acids synthesis and degradation. There are no reports describing the precise function of *ACSL6* in oocyte physiology, however it was shown that loss of ACSL fam-

ily member activity, in general, caused premature ovarian failure (POF) in *Xenopus laevis* by impairing meiotic arrest and speeding up oocyte maturation. It means that proper activity of ACSL synthetases may be responsible for maintaining physiological pool of arrested oocytes [26].

All genes belonging to fatty acid associated ontology groups showed significant down-regulation in porcine oocytes after IVM, in comparison to oocytes before IVM. Considering few information about these genes in relation to oocyte maturation, or even reproductive events, we cannot clearly correlate obtained results with mentioned processes. However, significant changes in gene expression point to their potential role as an oocyte developmental competence markers in pigs. Molecular diagnostics could be the prospective tool for selection of best gametes, thus leading to improved outcome of *in vitro* fertilization.

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Conflict of interest statement

The authors declare they have no conflict of interest

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