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Response to abiotic and organic substances stimulation belongs to ontologic groups significantly up-regulated in porcine immature oocytes

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Abstract

The efficiency of the process of obtaining mature oocytes, and then of porcine embryos in vitro depends on many factors and requires meeting many conditions. These include selection of morphologically appropriate oocytes, selection of appropriate medium components, as well as a number of abiotic factors (appropriate microenvironment during in vitro culture).

Oocytes were taken from 45 pubertal crossbred Landrace gilts. The BCB test was carried out. BCB + oocytes were divided into two groups: "before IVM" and "after IVM". "Before IVM" oocytes were subjected to molecular analyzes immediately after collection, while "after IVM" oocytes underwent in vitro maturation and then the second BCB test. Oocytes that remained BCB+ after the second test were used for molecular analyzes using Affymetrix expression microarrays.

A group of genes responsible for response to organic substance and response to abiotic stimulus, which underwent significant changes (decrease) was discovered after oocyte in vitro maturation. Genes such as MM, PLDP, SERPINH, MYOF, DHX9, HSPA5, VCP, KIT, SERPINH1, PLD1, and VCP showed the largest decrease after the culture period. The levels of these genes were therefore elevated in oocytes before the in vitro maturation process.

In conclusion, a number of organic and abiotic factors have an impact on the process of the oocyte in vitro maturation. The presented results confirm the literature data in which the low efficiency of obtaining mature oocytes in in vitro conditions is mentioned, which further impacts the amount of viable embryos obtained.

Running title: Response of porcine immature oocytes to abiotic and organic substances

Keywords: porcine oocytes, organic substances stimulation, abiotic stimulus

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Introduction

The procedure of *in vitro* pig embryo production consists of several very important, inseparable stages: oocyte procurement, oocyte *in vitro* maturation, oocyte fertilization and *in vitro* embryo culture [1].

The literature provides clear evidence that *in vitro* matured oocytes are significantly reduced in quality compared to oocytes maturing under physiological conditions. The reduced quality of acquired oocytes results in reduced survival and the ability to undergo fertilization. This result is a reduced number of embryos obtained [2].

The process of oocyte maturation in both physiological and *in vitro* conditions consists of many stages, with various underlying contributing factors.

Under physiological conditions, from the moment of recruitment of the primary follicle until the moment of ovulation, the process of oocyte maturation occurs. It involves the synthesis and accumulation of appropriate compounds and redistribution of cell organelles inside the oocyte, necessary for the process of fertilization and early embryo development [3]. During the aforementioned cytoplasmic maturation of the oocyte, it increases its volume (about 300 times) and the diameter (from 15μ m to 100 μm). In this time, the oocyte is very active. It is subject to the transcription and translation process, thanks to which it contains about 200 times more RNA and 20 times more proteins than the somatic cell. During the resumption of the meiosis process, the transcription stops and the translation process is continued [4].

The first stem of the nuclear maturation process of the oocyte is the resumption and completion of the first meiotic division, followed by the beginning of the second division and its arrest in the metaphase II stage [5]. During nuclear maturation, the nuclear envelope disappears, haploidization of the genetic material occurs and the first directional body is ejected. The physiological signal for the resumption of meiosis is the sudden increase in luteinizing hormone (LH). The effect of its action is a decrease in cAMP concentration in oolema, cAMP-dependent kinase activity inactivating maturation promoting factors (MPF) [6]. MPF is responsible for the regulation of mechanisms controlling meiosis and the entire cell cycle. The activity of this factor causes the inhibition of transcription and the continuation of the meiotic division [7].

It is believed that it is much harder in an *in vitro* environment to acquire full cytoplasmic maturity. In recent years, the conditions of *in vitro* oocytes cultures have been optimalized, thanks to which a large number of oocytes are obtained at the stage of metaphase II [8]. Many biotic and abiotic factors influence the process of oocytes maturation *in vitro*. First of all, it is the selection of appropriate oocytes, morphologically unchanged after the procurement process, with homogeneous cytoplasm, surrounded by a compact group of cumulus cells. Another very important factor is the selection of an appropriate culture medium. The medium should contain properly selected ingredients [8]. Also factors such as the culture microenvironment is very important during the *in vitro* maturation process [9,10]. The present article focuses on genes that show reduced expression after *in vitro* oocytes culture in response to organic substances and abiotic stimulus.

Material and Methods

Parts of the materials and methods were already described in a different work by our group [11].

Animals

A total of 45 pubertal crossbred Landrace gilts, with a median age of 170 days and weight of 98 kg, were used in this study. The animals were bred under the same conditions. Experiments were approved by the Local Ethics Committee in Poznan (Resolution No. 83/2012/DNT).

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

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 10 min. at 38o C. in 0.9% NaCl. To provide optimal conditions for subsequent oocyte maturation and fertilization in vitro, the ovaries of each animal were placed in 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS [12,13]. Thereafter, single large follicles (>5mm) were opened, in a sterile Petri dish, by puncturing with a 5-mL syringe and 20-G needle, to recover the COCs. 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). COCs were selected under an inverted microscope- Zeiss, Axiovert 35 (Lübeck, Germany), counted, and morphologically evaluated with special care, using the scale suggested by Pujol et al. and Le Guienne et al. [14,15].

Grade I: COCs with a homogeneous cytoplasm and a complete cumulus oophorus

Grade II: COCs with an incomplete but compact cumulus oophorus with more than five layers and a homogeneous cytoplasm

Grade III: COCs with a heterogeneous cytoplasm and more than three cumulus cells layers or more than five layers in some parts.

Grade IV: COCs with cumulus oophorus partly or totally absent and cytoplasm strongly heterogeneous."

Only COCs of grade I with homogeneous cytoplasm and uniform and compact cumulus cells were considered for use in the following steps of the experiment, resulting in the use of a total of 300 grade I oocytes that were determined positive in Brilliant Cresyl Blue (BCB) test.

Assessment of oocyte developmental competence by BCB test

Brilliant Cresyl Blue (BCB) test, which measures the activity of glucose-6-phosphate (G6PDH) enzyme, was used for assessment of oocytes' quality and maturity [16]. The G6PDH enzyme converts BCB stain from blue to colourless. In oocytes that completed their growth, the activity of the enzyme decreases and the stain cannot be reduced, resulting in blue oocytes (BCB+). To run the BCB staining test, oocytes were washed two times in modified Dulbecco PBS (DPBS) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 50 IU/mL penicillin, 50 µg/mL streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 0.4% [w/v] BSA, 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). Thereafter, they were treated with 13 µM BCB (Sigma-Aldrich, St. Louis, MO, USA) diluted in DPBSm at 38.5°C, 5% CO2 in air for 90 min. After treatment, the oocytes were transferred to DPBSm and washed two times. During the washing procedure, the oocytes were examined under an inverted microscope and classified as either having stained blue (BCB+) or remained colourless (BCB–) (**Fig. 1**). Immature oocytes have compact cumulus cell layers which need to be removed for further oocyte evaluation, regardless if the oocyte will be later analyzed as "before IVM" group or set to undergo IVM. Therefore, the BCB+ COCs were first incubated with bovine testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) for 2 min at 38o C, to separate cumulus and granulosa cells. Afterwards, cells were removed by vortexing the BCB+ oocytes in 1% sodium citrate buffer and by mechanical displacement using a small-diameter glass micropipette. Only the granulosa-cell-free BCB+ oocytes were used for subsequent IVM, microarray and RT-qPCR analysis. Overall, 150 immature oocytes were qualified as "before IVM", analyzed straight after the COC shedding, with the rest directed for in vitro maturation ("after IVM") and subsequent analysis.

In vitro maturation of porcine COCs

After the first BCB test, the blue stained COCs (BCB+) were cultured in Nunclon™Δ 4-well dishes 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 (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/mL eCG (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38° C, under 5% CO2, for 22h, and then for additional 22h in medium without hormones. After maturation, the BCB staining test was performed again and BCB+ oocytes were used for further molecular analyses. Based on visual analysis under an inverted microscope, around 70% of oocytes were determined as BCB+. This makes the final number of "after IVM" oocytes around 105 out of the initial 150.

RNA extraction from porcine oocytes

Total RNA was extracted from all the samples (both before and after IVM), using TRI Reagent (Sigma, St Louis, MO, USA) and RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). The amount of total mRNA was determined from the optical density at 260 nm, while 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 (Agilent Technologies, Inc., Santa Clara, CA, USA). 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, 100 ng of RNA was taken for the microarray analysis. The remaining amount of isolated RNA was used for RT-qPCR study.

Microarray expression analysis and statistics

The Affymetrix procedure was described previously in our papers [17,18]. cDNA was reverse transcribed from Total RNA (100 ng) (Ambion® WT Expression Kit; Ambion, Austin, TX, USA). Obtained cDNA was biotin labelled and fragmented by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix, Santa Clara, CA, USA). Biotin-labelled fragments of cDNA (5.5 µg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (48°C/20 h; Affymetrix, Santa Clara, CA, USA). Then, microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by Imaging Station of GeneAtlas System.

The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM Operating Software (Affymetrix, Santa Clara, CA, USA). The 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 of the presented analyses and graphs were performed using Bioconductor and R programming language. Each CEL file was merged with a description file. In order to correct background, normalize and summarize results, we used Robust Multiarray Averaging (RMA) algorithm.

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. All statistical analyses were performed using Statistica software (Version 13.3; StatSoft, Poland).

Differentially expressed genes were subjected to the selection of genes associated with a cellular component of morphogenesis. Differentially expressed gene lists (separate for up and down-regulated genes) were uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery; Leidos Biomedical Research, Inc., National Cancer Institute, Frederick, MD, USA). Such analysis allowed us to extract significantly enriched gene sets from Gene Ontology (GO) Biological Process (BP) database.

Subsequently set of differentially expressed genes from "response to abiotic stimulus" and "response to organic substances" GO BP terms, were applied to STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins; STRING Consortium) for interactions prediction. STRING is a huge database contains information about protein/gene interactions, including experimental data, computational prediction methods and public text collections.

The further investigation of the studied GO terms, we have calculated the z-score (the number of up-regulated genes minus the number of down-regulated genes divided by the square root of the count) analysis with GOplot package [19]. The results allowed us to investigate the enrichment of those two GO BP terms.

Ethical approval

The research related to animal use has been complied with all the relevant national regulations and instructional policies for the care and use of animals. Bioethical Committee approval no. 83/2012/DNT.

Results

Profiling of the whole transcriptome of the oocyte by Affymetrix microarray allowed us to analyze gene expression changes after *in vitro* maturation (after IVM) in relation to the freshly isolated oocyte, before *in vitro* procedure (before IVM). Using Affymetrix® Porcine Gene 1.1 ST Array, (available in GEO database, accession: GSE97246), we examined the expression of 12258 porcine transcripts (microarray data: supplementary materials). We considered genes of fold change higher than |2|, and with a corrected p-value lower than 0.05, as differentially expressed. This set of genes consists of 419 different transcripts. The first detailed analysis based on GO BP allowed us to identify 51 significantly enriched GO BP terms. Among them, we focused on from "response to abiotic stimulus" and "response to organic substances". The genes that belong to this term were subjected to hierarchical clusterization algorithm and presented as a heatmap graph (**Fig. 1**). The gene symbols, fold changes in expression, Entrez gene IDs and corrected p values of that genes were shown in **table 1**. The enrichment of

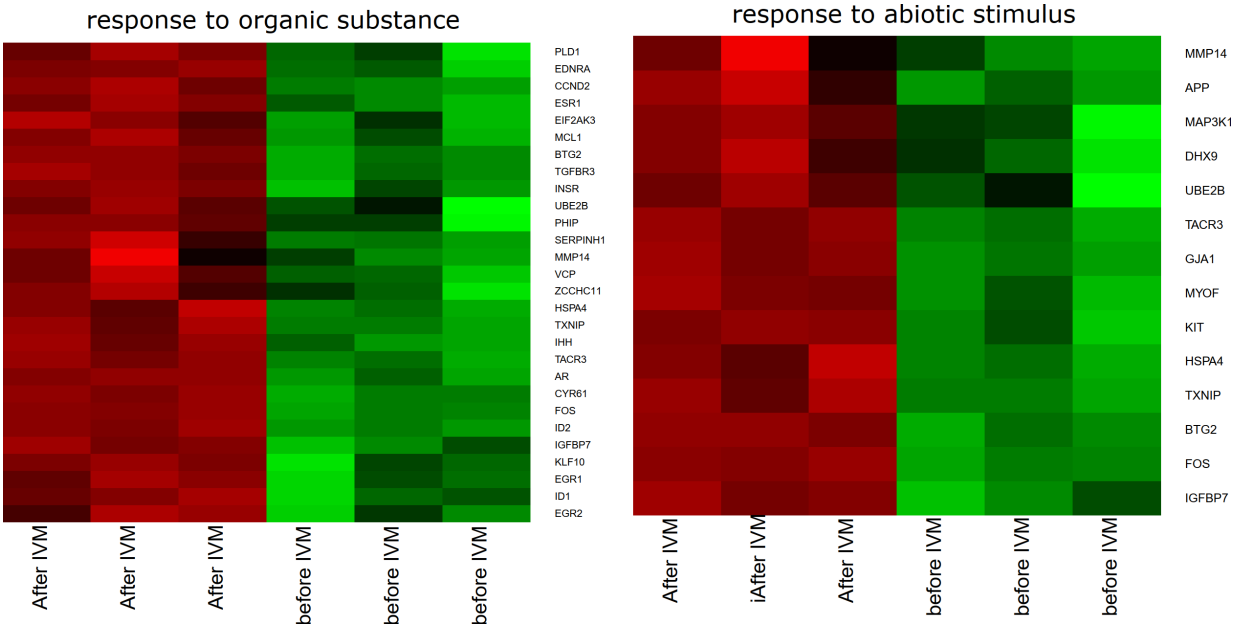


FIGURE 1 Heat map representation of differentially expressed genes belonging to the "response to abiotic stimulus" and "response to organic substances". GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower expression). Log2 signal 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)

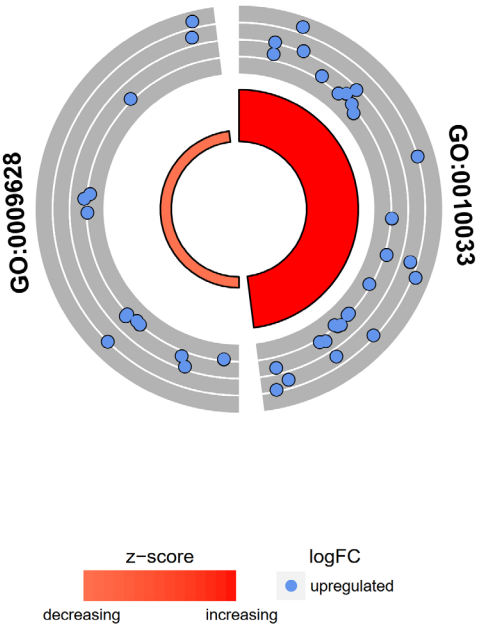
TABLE 1 Symbols, fold changes in expression, Entrez gene IDs and corrected p values of genes belonging to "response to organic substance" and " response to abiotic stimulus"

Official Gene Symbol	Fold Change	adjusted p.value	ENTREZ GENE ID
FOS	0,052794356	4,74E-05	2353
ID2	0,062979704	4,74E-05	3398
BTG2	0,074386393	9,55E-05	7832
CYR61	0,080657036	7,54E-05	3491
ESR1	0,081629841	0,000522187	2099
AR	0,1059863	0,000138367	367
TACR3	0,115060322	0,000148036	6870
CCND2	0,121809064	0,000178804	894
EGR2	0,165503832	0,007949861	1959
EDNRA	0,166939028	0,00185422	1909
TGFBR3	0,196522244	0,000405979	7049
GJA1	0,206907347	0,000107676	2697
MCL1	0,244179957	0,001775249	4170
IHH	0,304995843	0,000551261	3549
INSR	0,31601561	0,001912689	3643
ZCCHC11	0,3216223	0,019809962	23318
APP	0,324138605	0,005602323	351
ID1	0,335473139	0,003974331	3397
TXNIP	0,355538611	0,000780875	10628
MAP3K1	0,36876538	0,024748462	4214
EGR1	0,376128185	0,005477006	1958
UBE2B	0,382779667	0,041104659	7320
PHIP	0,385682339	0,02111605	55023
IGFBP7	0,403759522	0,002496043	3490
KLF10	0,405438718	0,00684513	7071
EIF2AK3	0,41888965	0,008422055	9451
KIT	0,430444215	0,00255635	3815
VCP	0,435612412	0,007402292	7415
HSPA4	0,441182204	0,002321468	3308
DHX9	0,44612444	0,022483263	1660
MYOF	0,458044533	0,002305619	26509
SERPINH1	0,467321273	0,006338248	871
PLD1	0,468341554	0,011044722	5337
MMP14	0,488721147	0,038060423	4323

each GO BP term was calculated and shown on the circle diagram (**Fig. 2**)

Moreover, genes that formed one particular GO group in the Gene Ontology database can also belong to other different GO term categories. For this reason, we explore the gene intersections between selected GO BP terms. The relation between those GO BP terms was presented as circle plot (**Fig. 3**) as well as a heatmap (**Fig. 4**).

STRING interaction network was generated among differentially expressed genes belonging to each of selected GO BP terms. Using such a prediction method provided us with a molecular interaction network formed between protein products of studied genes (**Fig. 5**). Finally, we investigated the functional interactions between chosen genes with REACTOME FIViz app to Cytoscape 3.6.0 software. The results were shown in (**Fig. 6**).



ID	Description
GO:0010033	response to organic substance
GO:0009628	response to abiotic stimulus

FIGURE 2 The circle plot showing the differently expressed genes and z-score of "response to abiotic stimulus" and "response to organic substances". GO BP terms. The outer circle shows a scatter plot for each term of the fold change of the assigned genes. Red circles display up- regulation and blue ones down- regulation. The inner circle shows the z-score of each GO BP term. The width of the each bar corresponds to the number of genes within GO BP term and the color corresponds to the z-score

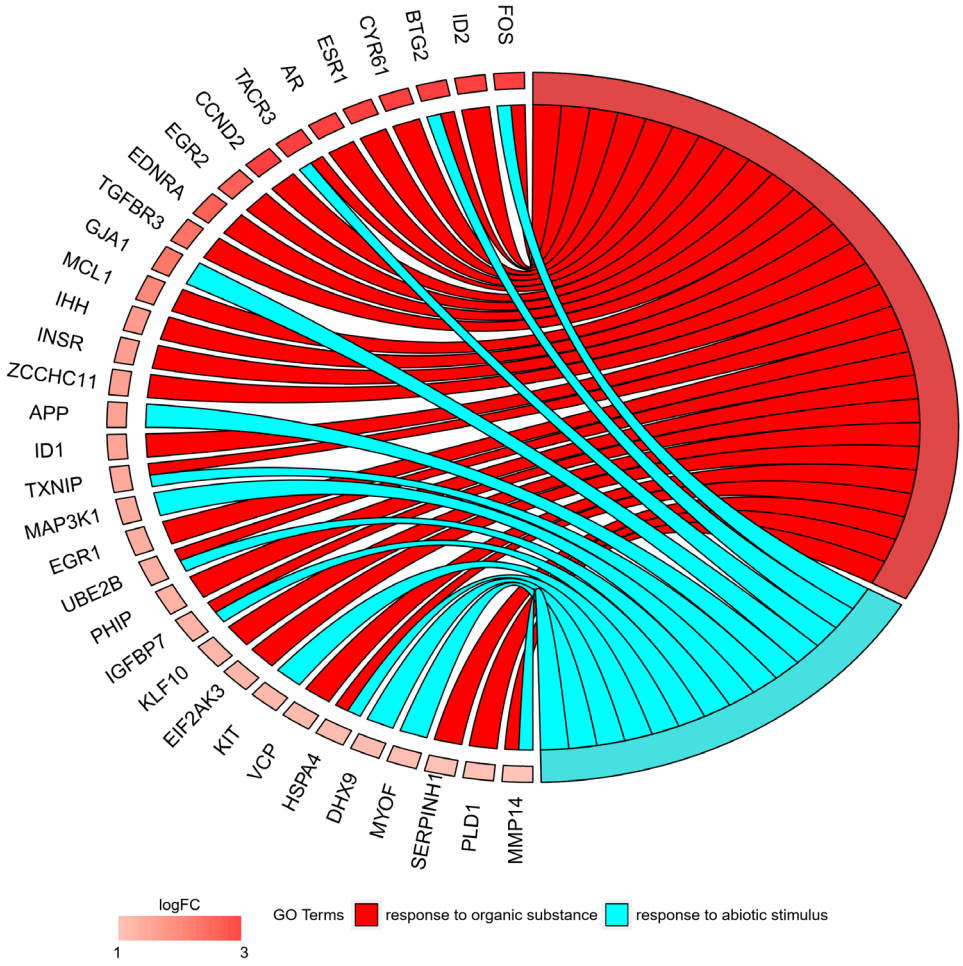


FIGURE 3 The representation of the mutual relationship between "response to abiotic stimulus" and "response to organic substances". GO BP terms. The ribbons indicate which gene belongs to which categories. The genes were sorted by logFC from most to least changed gene

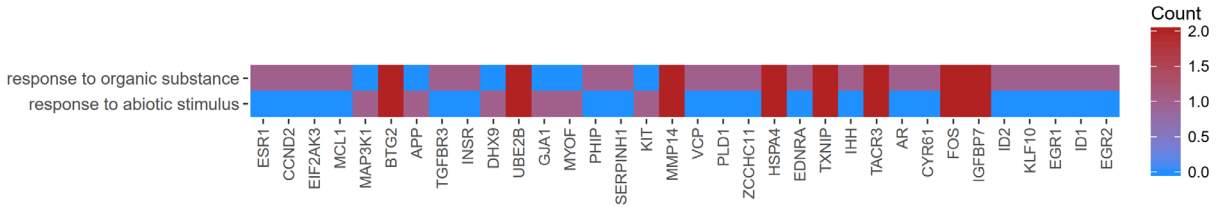


FIGURE 4 Heatmap showing the gene occurrence "response to abiotic stimulus" and "response to organic substances". GO BP terms

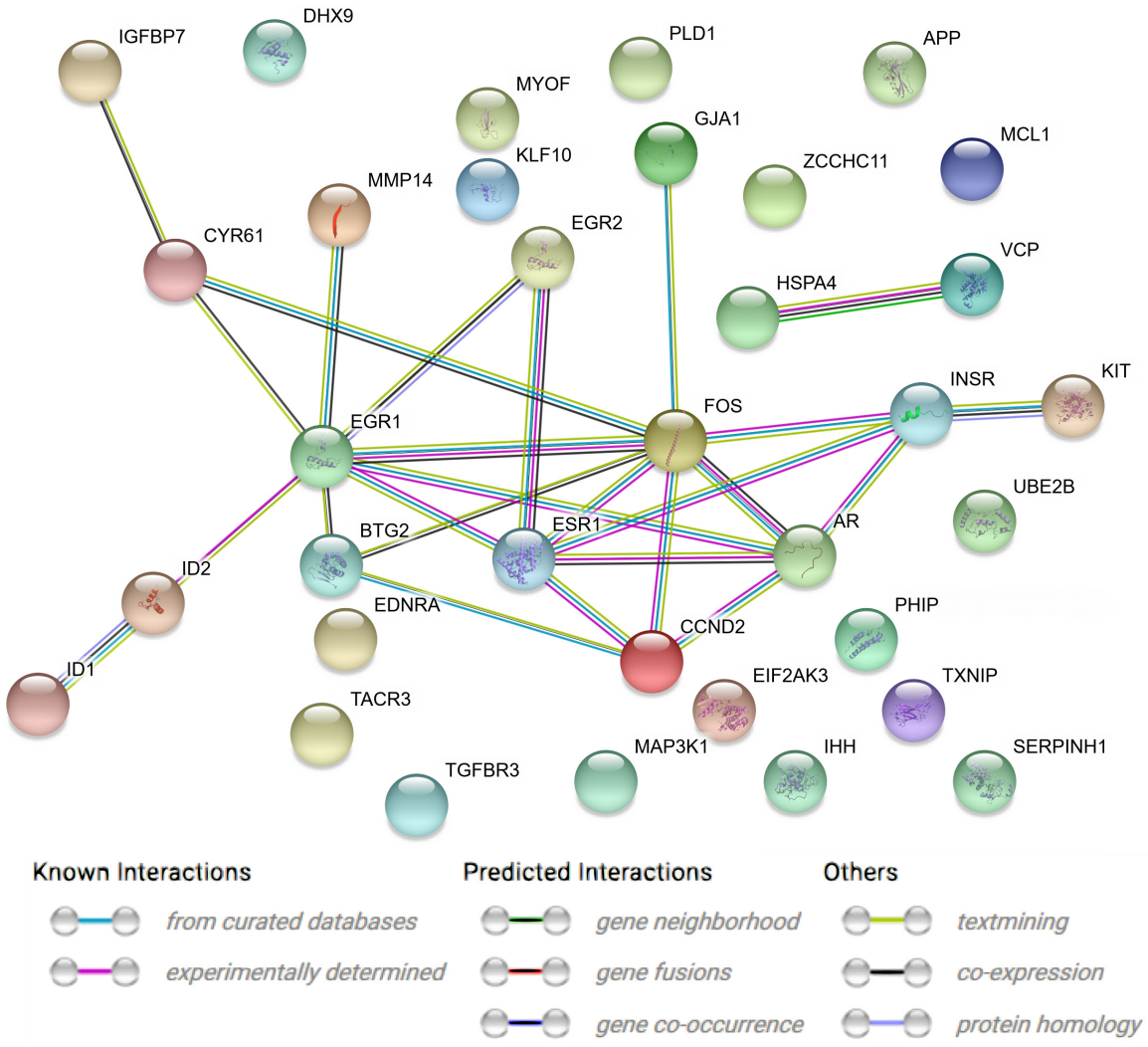


FIGURE 5 STRING-generated interaction network among differentially expressed genes belonging to the "response to abiotic stimulus" and "response to organic substances" GO BP terms. The intensity of the edges reflects the strength of interaction score

Discussion

In the presented article, we have analysed two groups of genes characterizing the response of oocytes to organic substance and abiotic stimulus from the external environment before *in vitro* culture and after *in vitro* culture.

The obtained results suggest that both of those groups of genes show significantly higher expression before IVM.

"Response to the organic substance" GO contains genes contributing to the processes that change the state or activity of a given cell (in terms of secretion, enzymatic activity, gene expression) under the influence of a specific organic substance. In turn, the "response to abiotic stimulus" ontological group describes a group of genes that causes a change in the state or activity of a cell under the influence of an abiotic factor.

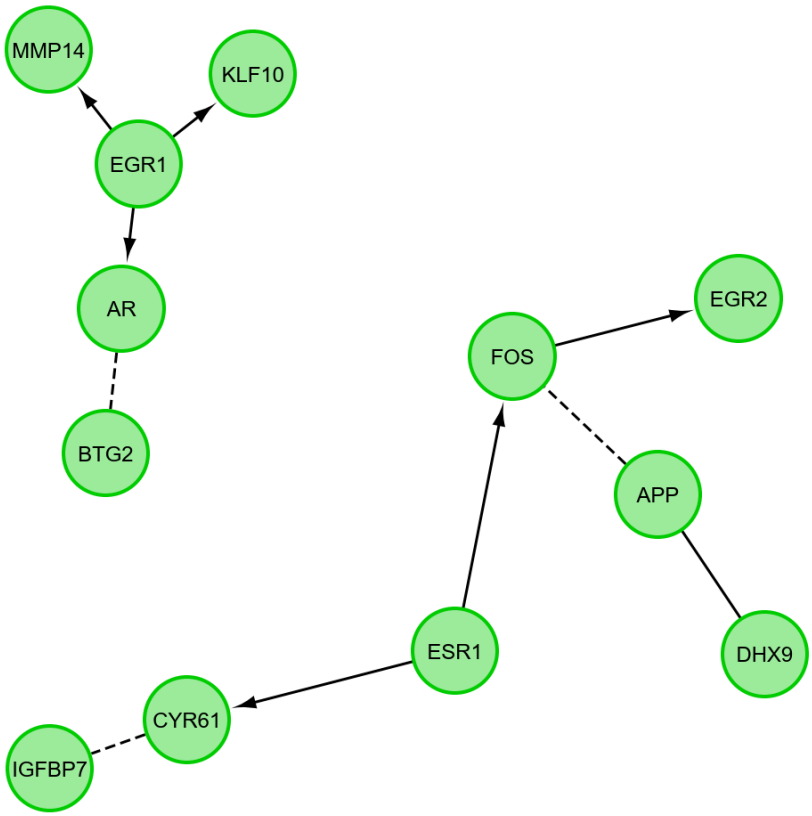


FIGURE 6 Functional interaction (FI) between differentially expressed genes belonging to the "response to abiotic stimulus" and "response to organic substances" GO BP terms. In following figure "->" stands for activating/catalyzing, "-|" for inhibition, "-" for FIs extracted from complexes or inputs, and "---" for predicted FIs

Genes such as *GJA1, APP, MAP3K1, KIT, DHX9, MYOF* - are characteristic for the "response to abiotic stimulus" ontological group and have been shown to increase their expression prior to oocyte culture. The change in these genes' expression drastically decreases after *in vitro* culture, and thus oocytes are less responsive to stimulation with abiotic factors after *in vitro* culture.

The first of presented gene, the *GJA1* (*Gap Junction protein alpha-1*) is a gene coding for membrane proteins - connexin (Cxs), primarily Cx43. Cxs is a group of membrane proteins that build slot channels that connect the cytoplasm of two neighbouring cells. This intercellular channel consists of 2 hemicalanals. Each such hemicanals consists of 6 Cx proteins. Cx43, as mentioned above, is present in many tissues and cells [22]. Many publications indicate that the *GJA1* gene represents a potential genetic marker associated with oocyte maturation and developmental competence of the obtained embryos [21,22].

Our studies have shown the association of *GJA1* with the *FOS* gene. In turn, *FOS* shows dependence with many other genes, as indicated by the STRING analysis. FOS has a critical function in regulating the development of cells designed to create a skeleton. It is also believed that FOS proteins are responsible for the proliferation, differentiation and transformation of cells [23]. This may concern even adipocytes [24]. We also know that the expression of genes from the FOS group (exactly c-FOS) is caused by extracellular stress [25].

The next gene is a *MAP3K1* (*Mitogen-Activated Kinase 1*). It is a mitogen-activated protein (MAP) kinase that regulates the MAPK and JNK pathways. *MAP3K1* is a factor that generates antiapoptotic signals, but may also induce apoptosis under certain conditions (after cleavage by caspase) [26]. *MAP3K1* is also involved in the process of cell migration. Expression of this gene was increased in oocytes before *in vitro* culture [27].

Increased expression prior to the *in vitro* culture of oocytes is also shown by the *Kit* gene (Stem cell factor receptor). KIT is necessary for the growth of germ cells both *in vivo* and *in vitro*. In addition, the Kit signalling pathway controls the proliferation of primary germ cells [28]. KIT is a receptor found in oocytes and theca cells. This gene also participates in the regulation of the process of folliculogenesis and oogenesis. KIT interacts with KIT LIGAND. The initiation of follicle growth from the original pool and their increase includes the KIT-KIT LIGAND interactions. During early ovarian follicle growth, *KIT* controls the growth of oocyte and cell differentiation, protecting the follicles against apoptosis. Also, the production of the antral cavity requires the effi-

cient functioning of the KIT-KIT LIGAND system. In mature ovarian follicles, this system is responsible for the ability of the ovum to mature its cytoplasmic components [29].

Another gene that has a higher expression before the oocyte *in vitro* culture is *MMP14* (*Matrix Metalloproteinase 14*). It is a gene characteristic for both of the analysed ontological groups. MMP14, as a member of the MMP protein family, is involved in the remodelling of the extracellular matrix associated with embryonic development. The MMP system, to which the MMP14 protein belongs, regulates many dynamic processes occurring in the ovary and uterus [30]. *KIT Ligand* (*KL*) is a product of granulosa cells in ovarian follicles. It is also believed to be a regulator of oocyte development. There is a hypothesis that the oocytes regulate the level of *Kit Ligand* mRNA expression. The development and growth of ovarian follicles are associated with an increase in the level of *KL* mRNA expression in granulosa cells. The presence of the oocyte in the culture caused an increase or decrease in the level of *KL* mRNA in granulosa cells depending on the absence or presence of FSH. It turns out, therefore, that the expression of the Kit gene depends on the presence of hormones in the culture, but also on the oocytes themselves that affect the granulosa cells [31].

SERPINH1, PLD1, and *VCP* have shown the highest expression among genes belonging to "response to organic substance" ontological group.

Ikegawa et al. noticed that *SERPIN H1* gene (*Serpin Peptidase inhibitor, clade H, member 1*) could respond and play an important role, together with retinoic acid, in the process of induced differentiation with into chondrocytes [32].

Another gene showing a high decrease after in vitro culture is *PLD1* (*Phospholipase D1*). The activity of PLD family proteins is associated with many cellular pathways (signal transduction, membrane transport). Hammond et al. showed that the protein has strong catalytic and regulatory properties. They were the first to suggest that PLD1 may affect the change in cell morphology as well as intracellular protein transport [33].

Conclusions

In this article, we focused primarily on genes that show the highest expression change. It turns out that they are involved in the processes associated with the proper functioning of the ovary and the maturation of the oocytes. In addition, genes belonging to the described ontological groups may be potential molecular markers of response to organic substances, as well as responses to abiotic factors. We could say that the expression of these genes increases / is high before oocyte culture *in vitro*. On the other hand, we can conclude that after *in vitro* culture the expression of these genes decreases drastically. This may indicate that during the change of abiotic factors, the oocytes reduce their compulsive competence in order to deescalate their reaction to those substances.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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