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Genes regulating biochemical pathways of oxygen metabolism in porcine oviductal epithelial cells during long-term IVC

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Abstract

Oxygen metabolism has an important role in the normal functioning of reproductive system, as well as the pathogenesis of female infertility. Oxidative stress seems to be responsible for the initiation or development of reproductive organ diseases, including polycystic ovary syndrome, endometriosis, preeclampsia, etc. Given the important role of maintaining balance between the production of ROS and antioxidant defence in the proper functioning of reproductive system, in the present study we aimed to analyse the expression of genes related to oxygen metabolism in porcine oviductal epithelial cells during long-term in vitro culture. The oviducts were collected from 45 crossbred gilts at the age of approximately nine months that displayed at least two regular oestrous cycles. The oviductal endothelial cells were isolated by enzymatic digestion to establish long-term primary cultures. Gene expression changes between 7, 15 and 30 daysof culturewere analysed with the use ofwhole transcriptome profiling by Affymetrix microarrays. The most of the "cellular response to oxidative stress" genes were upregulated. However, we did not observe any main trend in changes within the "cellular response to oxygen-containing compound" ontology group, where the gene expression levels were changed in various manner.

Running title: Oxygen metabolism in porcine oviductal epithelial cells

Keywords: porcine oviductal epithelial cells, long-term *in vitro* culture, oxygen metabolism, reactive oxygen species

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Introduction

Oxygen is an indispensable element of aerobic metabolism, playing a crucial role in cell respiration and influencing energy production. Oxygen atmospheric concentration is approximately 21%; however, mammalian cells are generally adapted to much lower oxygen levels. The oxygen supply is determined by the metabolic activity and functional status of organs and tissues, ranging from 2–9% *in vivo* [1,2]. An imbalance between the production of reactive oxygen species (ROS) and their detoxification leads to oxidative stress, resulting in cellular damage. The overproduction of ROS accompanied by impaired antioxidant system in cells could contribute to inflammation and senescence. The most destructive effect of ROS is their ability to cause single- and double-strand DNA breaks. Prolonged generation of ROS may have profound effects on epigenetic regulation of gene expression due to their interaction with several chromatin-modifying enzymes [2]. Moreover, ROS can lead to failure of membrane fluidity and function, as well as activation of apoptosis induced by lipid peroxidation of cell membrane or organelles [3]. Free radical-induced damage caused by oxidative stress plays a significant role in the pathogenesis of many diseases [4–7].

Oxygen metabolism has an important role in the normal functioning of reproductive system and in the pathogenesis of infertility in females. Oxidative stress seems to be responsible for the initiation or development of reproductive organ diseases, including polycystic ovary syndrome, endometriosis, preeclampsia, etc. [8–10]. However, disturbances in oxygen metabolism may take place outside of pathological conditions. Ovulation is considered to be a major stressor for surrounding cells, causing trauma-induced inflammation and unbalanced ROS levels, leading to local oxidative stress [11,12].

Animal oviducts and human Fallopian tubes represent a part of the female reproductive tract subjected to periodic exposure to free radicals. Whereas ROS contained in follicular fluid mainly affect the fimbriae, the backward flow of menstrual debris through the tubes, called menstrual reflux, may influence the oxygen tension along the entire uterine tube lumen. Exposure to free radicals, accompanied by local oxidative stress and inflammation, brings a risk of malignant transformation of Fallopian tube secretory epithelial cells, leading to high grade serous ovarian carcinoma (HGSC).The proper functioning of cellular antioxidant system helps to decrease the negative effects of local stressors, such as pelvic inflammation or retrograde menstruation-derived Fe^{3+}, and may contribute to prevention of cancer development [11,13].

Moreover, it has become clear that the oxygen levels influence fertilization and pre-implantation development of the embryo. The oviductal epithelial cells play an important rolein these processes by providing appropriate microenvironment within the uterine tube. Oviductal secretory fluid protects gametes from environmental stress to ensure embryo quality and positive pregnancy outcome [14,15]. The activity of antioxidant enzymes in oviductal epithelial cells is crucial for oxidative stress avoidance and contributes to the maintenance of low oxygen concentration within the uterine tube. Multiple meta-analyses have demonstrated improved pregnancy rates and live birth rates at 5% oxygen in the case of *in vitro* fertilization. While the mechanisms underlying the reduced developmental efficiency of embryos cultured at higher oxygen concentrations are not well understood, oxidative stress is supposed to be the potential cause of suboptimal embryo development.

However, a majority of clinical embryology studies have been performed at atmospheric oxygen concentrations, which may be explained by the additional cost associated with reducing the oxygen level in the culture environment [16].

Here, using the microarray approach, we aimed to investigate whether the atmospheric oxygen level could affect the expression of genes related to oxygen metabolism in porcine oviductal epithelial cells during long-term *in vitro* culture.

Material and Methods

Tissue Collection

In this study, oviducts were collected from 45 crossbred gilts, obtained from a commercial herd. The animals were at the age of approximately nine months and displayed at least two regular oestrous cycles. After reaching the anoestrus phase of the cycle, the animals were slaughtered. Collected oviducts were immediately transported to the laboratory within 30 min, kept in an isolated container at 38 °C.

Primary Long-Term Cell Culture

The method used for OEC harvesting and culture has been described in our previous studies [17–19]. Briefly, collected oviducts were washed twice in Dulbecco's phosphate buffered saline (PBS) and sectioned longitudinally. Then, OECs were gently scratched with surgical blades and subsequently digested for 1 h at 37 °C in 1mg/mL solution of collagenase I (Sigma Aldrich, St. Louis, MO, USA) in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich). The cell suspension was filtered through 40 µm pore size strainer and centrifuged for 10 min at 200× g. After rinsing with PBS, OECs were incubated for another 10 min at 37°C with 0.5% Trypsin/EDTA (Sigma Aldrich), filtered and centrifuged as described above, resuspended in supplemented DMEM (10% FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, and 1 µg/mL amphotericin B) and seeded onto culture dishes. The cells were cultured for up to 30 days (37 °C, 5% CO_2). The culture medium was changed every three days. Once OECs

reached confluence of around 75-80%, they were digested with 0.025% Trypsin/EDTA(Cascade Biologics, Portland, USA),centrifuged and passaged to another culture dish at a seeding density of 2×10^4 cells/cm^2.

RNA Isolation from Oviductal Epithelial Cells

OECs were suspended in TRI Reagent (Sigma Aldrich) after 24 h, 7, 15, and 30 days of culture. The total mRNA isolation was performed with the use of RNeasy MinElute cleanup Kit (Qiagen, Hilden, Germany). The quantity of isolated mRNA was assessed by measuring the absorbance at a wavelength of 260 nm, whereas the purity of RNA samples was determined as 260 nm/280 nm ratio of absorbance using a spectrophotometer (Bioanalyzer 2100, Agilent Technologies, Inc., Santa Clara, CA, USA).Each mRNA sample was diluted to 100 ng/μL concentration.

Microarray expression analysis and statistics

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

All of the presented analyses and graphs were generated using Bioconductor and R programming languages. Each CEL file was merged with a description file. To correct background, normalize, and summarize the results, we used the Robust Multiarray Averaging (RMA) algorithm. To determine the statistical significance of the analyzed genes, moderated t-statistics from the empirical Bayes method were performed. The obtained p-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate. The selection of significantly altered genes was based on a p-value beneath 0.05 and expression higher than two-fold.

Differentially expressed genes were subjected to selection through examination of genes involved in cell migration regulation. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to the DAVID software (Database for Annotation, Visualization and Integrated Discovery) [20], where genes belonging to the terms of all three Gene Ontology (GO) domains were extracted. Expression data of these genes were also subjected to the hierarchical clusterization procedure, with their expression values presented as a heatmap.

Subsequently, we analyzed the relation between the genes belonging to chosen GO terms with the GOplot package [21]. The GoPlot packagecalculated the z-score: the number of up- regulated genes minus the number of down- regulated genes divided by the square root of the count. This information allowed to estimate the change course of each gene-ontology term.

Interactions between differentially expressed genes/proteins belonging to the studied gene ontology group were investigated using the STRING10 software (Search Tool for the Retrieval of Interacting Genes) [22]. The list of gene names was used as a query for interaction prediction. The search criteria were based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression, and experimentally observed interactions. The results of such analyses generated a gene/protein interaction network where the intensity of the edges reflected the strength of the interaction score.

Finally, the functional interaction between genes that belong to the chosen GO BP terms were investigated with the REACTOME FIViz application to the Cytoscape 3.7.2 software. The Reactome FIViz app is designed to find pathways and network patterns related to cancer and other types of diseases. This application accesses the pathways stored in the Reactome database, allowing to perform pathway enrichment analysis for a set of genes, visualize hit pathways using manually laid-out pathway diagrams directly in Cytoscape, and investigate functional relationships among genes in hit pathways. The application can also access the Reactome Functional Interaction (FI) network, a highly reliable, manually curated pathway-based protein functional interaction network covering over 60% of human proteins.

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. 32/2012.

Results

Whole transcriptome profiling with Affymetrix microarrays allows us to analyze the gene expression changes between 7, 15 and 30 days of porcine oviductal epithelial cell culture. Using Affymetrix® Porcine Gene 1.1 ST Array Strip we examined the expression of 12257 transcripts. Genes with fold change higher than abs (2) and with corrected p-value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 2533 different transcripts.

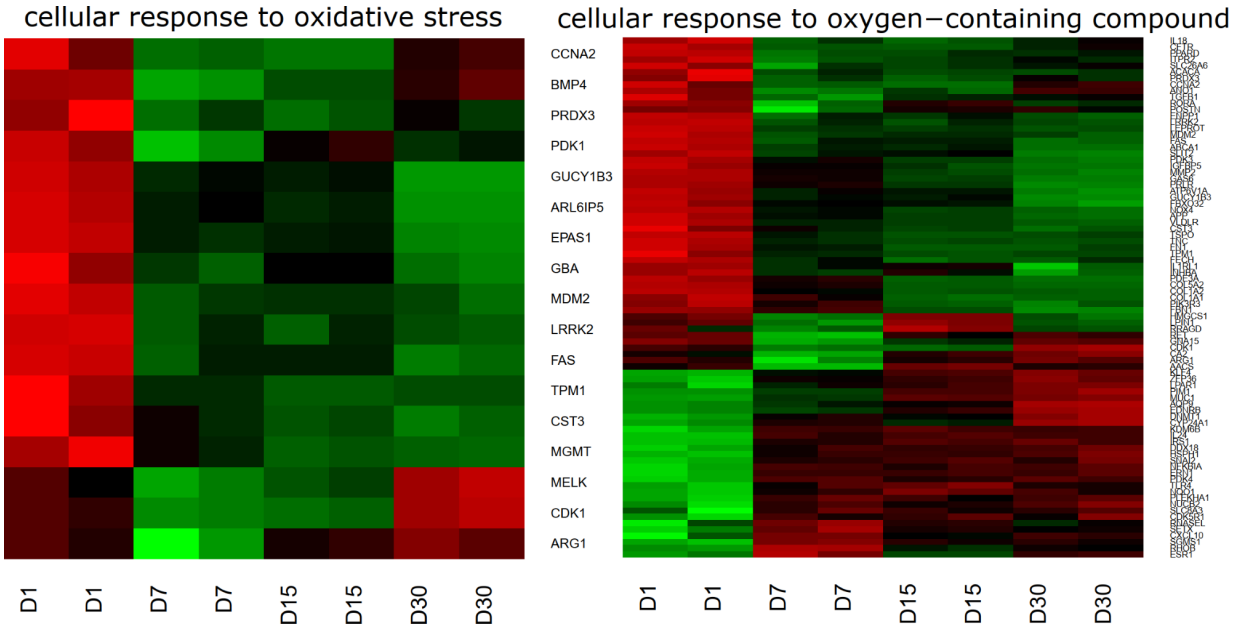

FIGURE 1 Heat map representation of differentially expressed genes belonging to the "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower 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). The 20 genes selected to this publication was marked green for upregulation and red for downregulation

TABLE 1 Gene symbols, fold change in expression ratio, Entrez gene IDs, corrected P values and mean value of fold change ratio of studied genes

GENE SYMBOL	RATIO D7/D1	RATIO D15/D1	RATIO D30/D1	ADJUSTED P VALUE D7/D1	ADJUSTED P VALUE D15/D1	ADJUSTED P VALUE D30/D1	ENTREZ GENE ID	MEAN RATIO
CCNA2	4,875266	5,344455	1,972133	0,000650	0,000366	0,025215	1E+08	4,063951
PRDX3	2,101021	2,183425	1,811495	0,002089	0,001295	0,004091	1E+08	2,031980
MDM2	2,436843	2,232734	2,561958	0,000183	0,000204	6,09E-05	1E+08	2,410512
FAS	4,208213	3,506662	5,536941	0,000131	0,000167	2,24E-05	396826	4,417272
TPM1	3,109678	3,824899	3,653385	0,000582	0,000183	0,000155	1E+08	3,529321
CST3	3,632418	5,335589	6,556542	0,003305	0,000725	0,000286	733672	5,174850
CDK1	4,809945	3,890205	-2,435634	0,000074	0,000100	0,000620	1E+08	2,088172
ARG1	4,347305	1,136755	-1,335509	0,000869	0,698683	0,310832	1,01E+08	1,382850

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of gene ontology biological process terms (GO BP) that contain differentially expressed transcripts. Up and down regulated gene sets were subjected to DAVID searches separately, with only gene sets with adj. p-value lower than 0.05 selected. The DAVID software analysis showed that the differently expressed genes belonged to 657 Gene ontology terms. In this paper, we focused on 90 genes that belong to "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (**Fig. 1**). The gene symbols, fold changes in expression, Entrez gene IDs and corrected p-values of these genes were presented in **table 1**.

The enrichment of each GO BP term was calculated as a z-score and shown on the circle diagram (**Fig. 2**).

The chosen GO BP terms contain 90 differently expressed genes. Therefore, we calculated the mean fold change of each gene between 7, 15 and 30 days of culture. Based on that criteria, we choose 10 most downregulated and 10 most upregulated genes to further analysis.

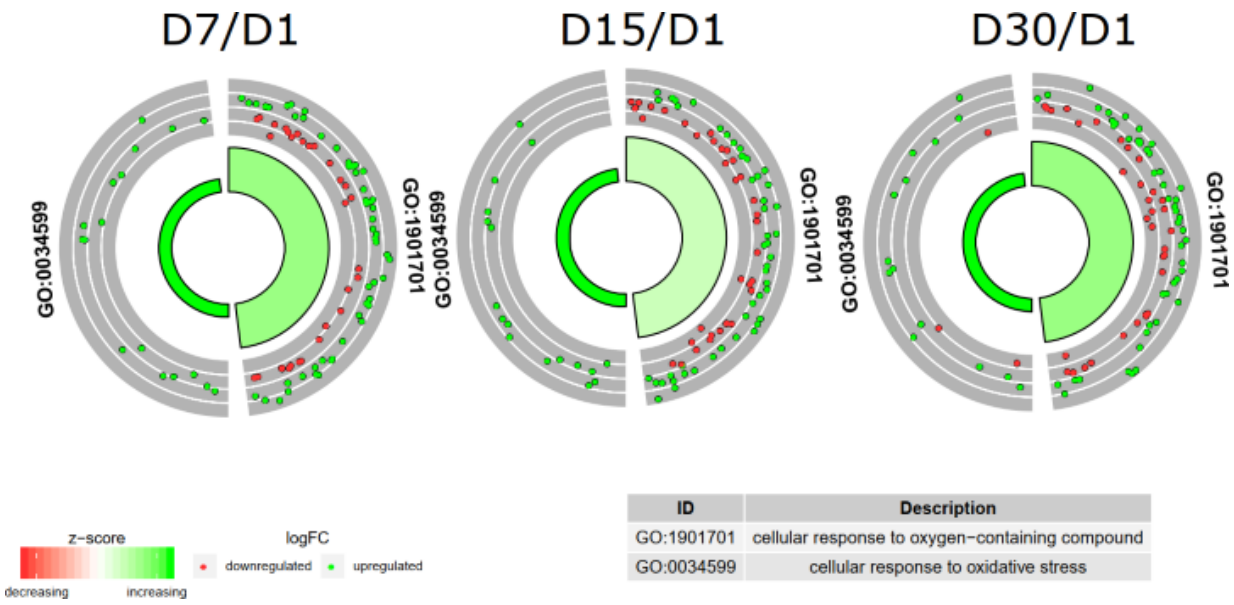

FIGURE 2 The circle plot showing the differently expressed genes and z-score of the "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. The outer circle shows a scatter plot for each term of the fold change of the assigned genes. Green circles display up-regulation and red 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

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

STRING-generated interaction network among differentially expressed genes belonging to each of selected GO BP terms. Using such 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 the chosen genes with the REACTOME FIViz app to the Cytoscape 3.7.2 software. The results were shown in (**Fig. 6**).

Discussion

Despite the essential role of oxygen for aerobic organisms, it may cause harmful reactions such as oxidative injury and senescence in living cells. ROS, produced by hyperoxic conditions, appear to be directly associated with the aging process. Cells cultivated under hyperoxic conditions show characteristics of cellular senescence, including growth arrest, lipofusin accumulation and protein oxidation [23,24]. All this leads to mitotic arrest, appearing to be caused by ROS-triggered malfunction of the cell cycle machinery [25].

Given the important role of maintaining the balance between the production of ROS and antioxidant defence in the proper functioning of reproductive system, in this study we have analysed the expression of genes related to oxygen metabolism in porcine oviductal epithelial cells during long-term culture. The genes belonging to "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms were differently expressed. However, most of the genes belonging to the former ontology group were upregulated after the 7 days days of culture, when compared with the first day. This may be due to the cell exposure to atmospheric oxygen tension, which is much higher than in oviduct, and may cause excessive ROS accumulation in culture medium *in vitro* [26].

Here, we would like to focus on eight genes belonging to both analyzed groups: *CCNA2, CDK1, PRDX3, MDM2, FAS, TPM1, CST3* and *ARG1*. The first two genes - *CCNA2* and *CDK1*–code proteins necessary for cell cycle progression through checkpoints. Cyclins, such as CCNA2, form complexes with cyclin dependent kinases (CDK), making the transition to the next phase of cell cycle possible. This promotes cell growth, unless inhibited by tumor suppressors, such as p53 or CDK-inhibitors (CDKi's), such as p21 or p27. Over expression of cell cycle inducers, such as the CDK's and cyclins, and under-expression of inhibiting factors are frequently found in tumors. Thus, Horree et al. reported a significantly higher expression of cyclin A in tubal metaplasia compared to the normal endometrium [27]. *CDK1*gene encodes a protein binding to all cyclins, which results in the phosphorylation of the Rb protein and the expression of genes regulated by E2F transcription factors. In the absence of CDK1, the embryos fail to develop into the morula and blastocyst stages. Therefore, CDK1 has been suggested to be the only essential cell cycle CDK [28].

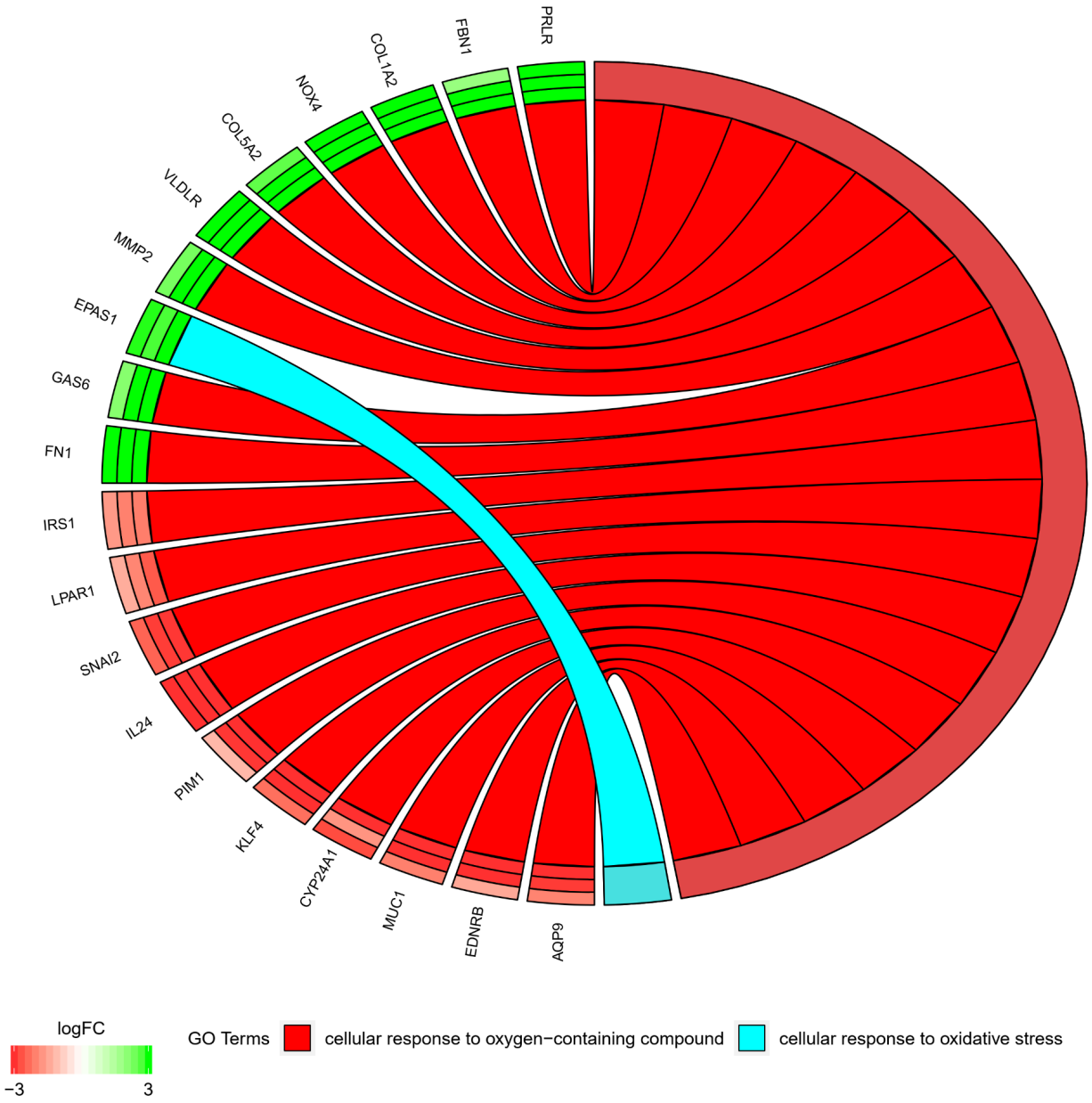

FIGURE 3 The representation of the mutual relationship of differently expressed genes that belongs to 20 chosen genes from "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. The ribbons indicate which gene belongs to which categories. The middle circle represents logarithm from fold change (LogFC) between D7/D1, D15/D1 and D30/D1 respectively. The color of each block corresponds to the LogFC of each gene (green – upregulated, red – downregulated). The genes were sorted by logFC from most to least changed gene

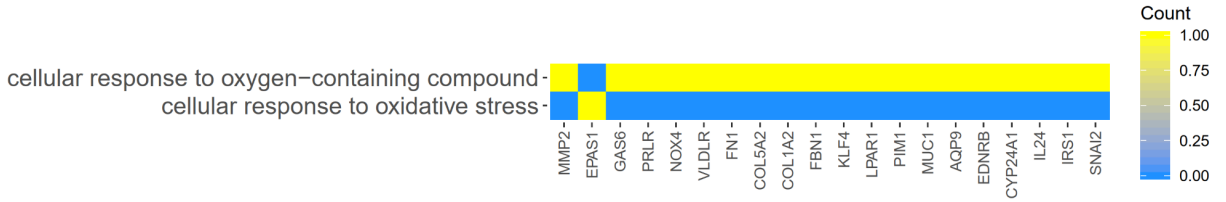

FIGURE 4 Heatmap showing the gene occurrence between chosen 20 differently expressed genes that belongs to "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. The yellow color is associated with gene occurrence in the GO Term. The intensity of the color is corresponding to amount of GO BP terms that each gene belongs to

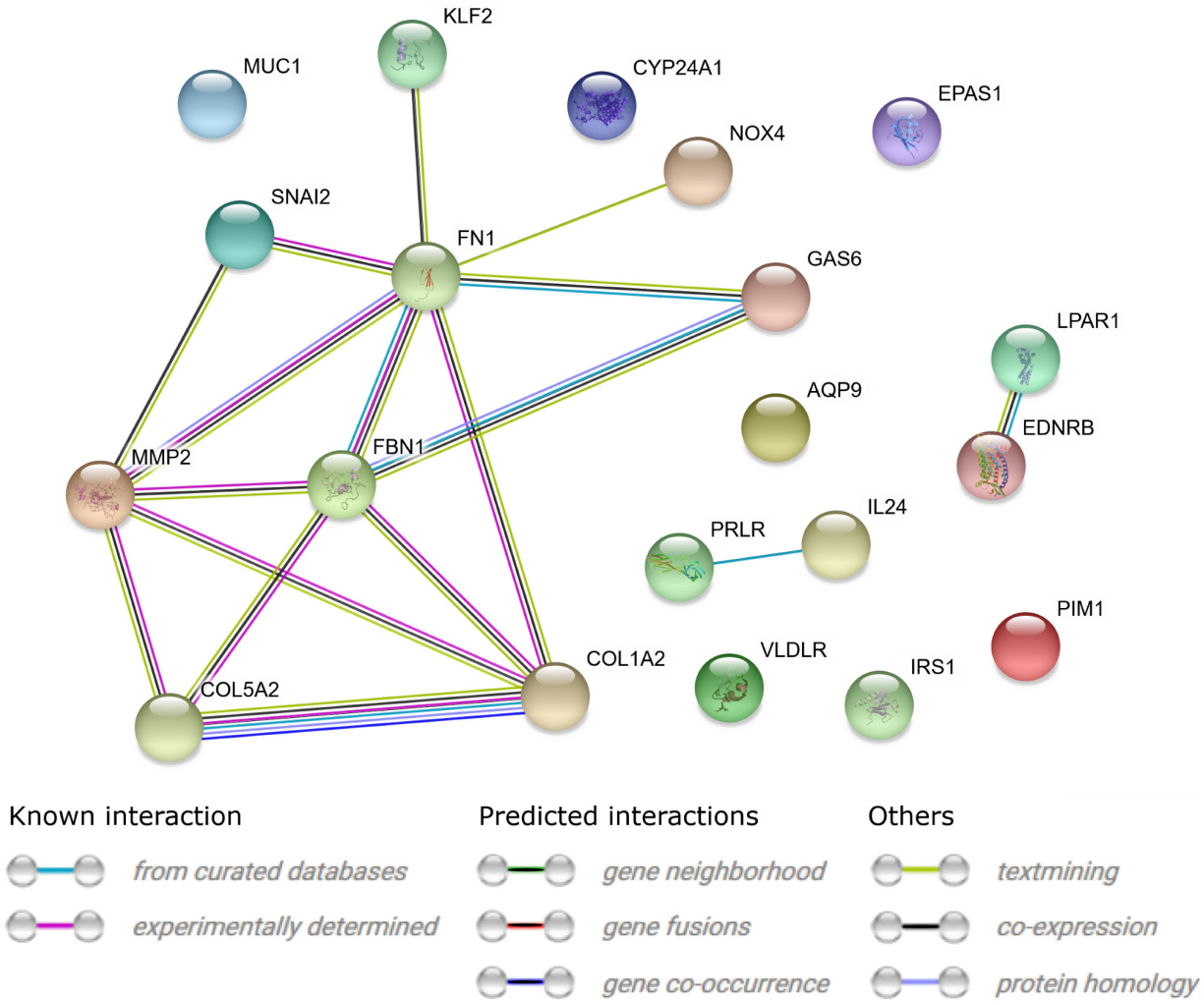

FIGURE 5 STRING-generated interaction occurrence between 20 chosen differently expressed genes that belongs to the "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. The intensity of the edges reflects the strength of interaction score

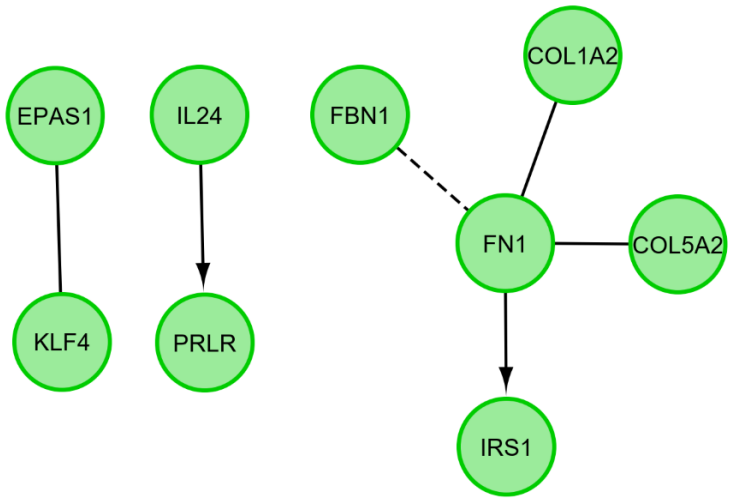

FIGURE 6 Functional interaction (FI) between 20 chosen differently expressed genes that belongs to the "cellular response to oxidative stress" and "cellular response to oxygen-containing compound" GO BP terms. In following figure "->" stands for activating/catalyzing, "-|" for inhibition, "-" for FIs extracted from complexes or inputs, and "---" for predicted FIs

In our study, *CCNA2* and *CDK1* genes exhibited the similar pattern of expression during the culture – they were up-regulated after 7 and 14 days and down-regulated at D30. We suggest that down-regulation of *CCNA2* and *CDK1* could be associated with the signs of cellular senescence resulting from long-term culturing. Furthermore, we have also observed down-regulation of the*ARG1* gene, which has been reported to possess anti-inflammatory properties, at D15-D30 [29].

The next analyzed gene, *PRDX3*, encodes an antioxidant enzyme that modulates cellular response to reactive oxygen species. PRDX3 enzyme is localized exclusively in mitochondria. It has been demonstrated that PRDX3 functions in parallel with mitochondrial glutathione peroxidases to protect mitochondria against oxidative injury by scavenging H_2O_2 [30,31]. The study of Bonfim et al. revealed that bovine blastocysts under high oxygen partial pressure exhibited elevated ROS accumulation and increased expression of *PRDX1* and *PRDX3*,confirming the correlation between oxidative stress level and expression of the aforementioned genes [32].

We have also observed up-regulation of the *MDM2* gene, which encodes an oncoprotein targeting the p53 tumor suppressor for proteasomal degradation [33,34]. Due to the ability to inhibit p53 activity, MDM2 promotes cell survival and cell cycle progression.

TPM1 is the next gene belonging to both studied ontology groups, which was upregulated after D7 of culture. *TPM1*plays a key role in cytoskeleton remodelling and stress fibre formation in response to oxidative stress [35]. On the other hand, there have been studies demonstrating that *TPM1* functions as a tumour suppressor gene in chosen tumour cell lines [36]. In our recent research, we have found that TPM1 was involved in cell migration mechanisms and up-regulated in porcine oocytes before *in vitro* maturation(IVM) as compared with post-IVM expression analysis [37].

Interestingly, the obtained data revealed upregulation of genes encoding the proteins, which ensure the optimal conditions for spermatozoa transfer through the female reproductive tract, as well as fertilization. These genes are cell surface death receptor (*FAS*) and cystatin (*CST3*). The former is involved in the processes ensuring that spermatozoa in the tubal sperm reservoir escape phagocytosis by polymorphonuclear leukocytes. This may be due to the ability of the Fas–FasL system to eliminate cytotoxic T lymphocytes and NK cells to prevent them from attacking spermatozoa or the early embryo in the oviduct [38]. The second gene - *CST3* – encodes a protein prominently expressed in the female reproductive tract, including the epithelial lining, particularly at times around ovulation.CST3 may help spermatozoa enter the upper reproductive tract by enhancing their motility [39].

In conclusion, our study revealed that atmospheric oxygen affects the expression of genes involved in oxygen metabolism. The most of the "cellular response to oxidative stress" genes were upregulated during the culture. However, we did not observe any main trend in changes within the "cellular response to oxygen-containing compound" ontology group, where the gene expression levels were changed in various manners.

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Conflict of interest statement
The authors declare they have no conflicts of interest.

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