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Analysis of fructose and mannose – regulatory peptides signaling pathway in porcine epithelial oviductal cells (OECs) primary cultured long-term *in vitro*

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Abstract

The morphological and biochemical modification of oviductal epithelial cells (OECs) belongs to the compound process responsible for proper oocytes transport and successful fertilization. However, the main mechanisms which regulated this process are still not entirely known. Moreover, the OECs metabolism, which may be identified as the "cellular activity" marker, is poorly recognized. In this study we investigated the fructose and mannose metabolic pathway in porcine OECs primary long-term cultured in vitro.
In our study, we employ a primary long term in vitro culture (IVC) and microarray approach (the Affymetrix microarray were used for analysis of transcriptomic profile of OECs) for expression levels analysis.
We found that from the whole analyzed transcriptome, 1537 genes were upregulated and 995 were down regulated after 7 days of culture, 1471 genes were upregulated and 1061 were downregulated after 15 days of culture and 1329 genes were upregulated and 1203 were downregulated after 30 days of culture. Moreover, the differential expression of SORD, FPGT, PFKFB4, TPI1, MPI, ALDOC, HK2 and PFKFB3 at 24 hours, 7 day, 15 day and 30 day, was also observed.
We suggested that fructose and mannose metabolism may be important molecular bio-marker of porcine OECs capability in in vitro model. The metabolic profile is significantly accompanied by cells proliferation in vitro. The transcriptomic profile of SORD, FPGT, PFKFB4, TPI1, MPI, ALDOC, HK2 and PFKFB3 expression may be identified as "fingerprint" of fructose and mannose metabolism in OECs as well as involved in cellular in vitro developmental capacity in pigs.

Running title: Regulation of fructose and mannose metabolism

Keywords: pig, OECs, microarray, in vitro culture (IVC)

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Introduction

Recently a lot of experiments were developed based on primary cell culture models, including several species of mammals, organs and tissues. The results from these studies revealed the differences in cells proliferation ability in vitro, which is significantly related to the type of tissue originated cells. Additionally, the cells revealed various differentiation capability in vitro to the others, often distinguish cell types. It was found that some of these long-term cultured primary cells in vitro are characterized by huge transdifferentiation capacity, which may be a key factor in application of these cells population and/or structurally advanced tissue as the grafts in regenerative medicine in both human and animals. Therefore, the recent research based on cells and/or tissues cultured primary is of high interest and significance.

Our recent studies (unpublished data) indicated possible primary in vitro cultivation of several toes of cells originated from reproductive organs in human and animals. Indeed, we introduced the primary culture systems based on: (1) human ovarian granulosa cells, (2) porcine ovarian granulosa cells, (3) porcine endometrial and myometrial cells, and (4) porcine epithelial oviductal cells (OECs) [1-5].

The porcine OECs are received from slaughterhouse and then isolated microsurgically, mechanically, and enzymatically. The collected epithelial tissue after separation was may be successfully transferred to culture system for short,- and/or long-term cultivation. Our experiments indicated that OECs were successfully kept in primary culture system for long-term with several passages, leading to cells morphological and biochemical modification. Besides the OEC causing an increased proliferation ability, they may also differentiate into other cell types. Although the procedures of isolation of oviductal epithelium is well recognized in several species of mammals, the primary in vitro cultivation of OECs, is presented by our team in pigs for the first time.

The gene expression profile in OECs primary cultured for a long-time using microarray assays is demonstrated in this study for the first time. Using Affymetrix microarray assays, we have shown the regulatory peptides and enzymes involved in fructose and mannose signalling pathways. In this study we demonstrated the role of the proteins involved in these sugars metabolism during OECs long-term primary culture in vitro, for the first time.

Material and Methods

Animals

In this study, crossbred gilts (n=45) at the age of about nine month and which displayed two regular estrous cycles were collected from a commercial herd. All the animals were checked daily for estrus behavior and were slaughtered after reaching the anestrus phase of the estrus cycle. The uteri were then transported to the laboratory within 30 min at 38 °C.

Oviductal epithelial cells (OECs) selection and culture

Oviducts were washed twice in Dulbecco's phosphate buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Epithelial cells were surgically removed using sterile surgical blades. Then, the epithelium was incubated with collagenase I (Sigma Aldrich, Madison, USA), 1mg/mL in Dulbecco's modified Eagle's medium (DMEM; Sigma Aldrich, Madison, USA) for 1 h at 37 °C. The cell suspension obtained from this digestion was filtered through 40 µm pore size strainer to remove blood and single cells. The residue was collected by rinsing the strainer with DMEM. Cells were centrifuged (200 g, 10 min.). Next, they were washed in PBS and centrifuged. Later, they were incubated with 0.5% Trypsin/EDTA (Sigma Aldrich, Madison, USA) at 37 °C for 10 min. The reaction was stopped with fetal calf serum (FCS; Sigma Aldrich, Madison, USA). After incubation, cells where filtered and centrifuged again. The final cell pellet was suspended in DMEM supplemented with 10% FCS, 100U/mL penicillin, 100 µg/mL streptomycin and 1µg/mL amphotericin B. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO^2. Once the OEC cultures attained 70–80% confluency, they were passaged by washing with PBS, digested with 0.025% Trypsin/EDTA, neutralized by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifuged, and resuspended at a seeding density of $2x10^4$ cells/cm^2. The culture medium was changed every three days.

RNA extraction from Oviductal epithelial cells (OECs)

Oviductal epithelial cell were pooled and harvested 24h, 7days, 15 days and 30 days after beginning of culture. 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 from the 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 (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, 500 ng of RNA was taken for microarray expression assays.

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 by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). 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 Imaging Station of the GeneAtlas System. Preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM 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 performed using Bioconductor and R programming languages. Each CEL file was merged with a description file. In order to correct background, normalize, and summarize 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 selection by examination of genes involved in cell migration regulation. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery) [6], where genes belonging to "Fructose and mannose metabolism" pathway from Kyoto Encyclopedia of Genes and Genomes (KEGG) were obtained. Subsequently the expression levels of that genes were marked on the pathways using a "pathview" - tool set for pathway based data integration and visualization [7]. Expression data of these genes were also subjected to a hierarchical clusterization procedure, and their expression values were presented as a heat map.

Interactions between differentially expressed genes/proteins belonging to the "fructose and mannose" KEGG pathway were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes) [8]. The list of gene names was used as a query for an 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.

Results

To investigate oocyte transcriptome changes following 7, 15 and 30 days after start of primary porcine oviductal epithelial cells, we performed whole gene expression analysis by Affymetrix® Porcine Gene 1.1 ST Array. In such assay expression of more than 14789 porcine transcripts was examined. The genes for which the fold change was higher than the cut-off value (fold>|2|) and corrected p value <0.05, were considered as differentially expressed. From the whole transcript that consist of 2552 different genes. From these genes 1537 were upregulated and 995 were down regulated after 7 days of culture, 1471 were upregulated and 1061 were downregulated after 15 days of culture and 1329 were upregulated and 1203 were downregulated after 30 days of culture.

Among these genes, genes belong to "fructose and mannose metabolism" KEGG pathway were extracted by DAVID (Database for Annotation, Visualization and Integrated Discovery) software. Subsequently the logarithm from expression values of these genes were marked on these pathways using "pathview" tools set (Fig. 1). Up and down regulated gene sets were subjected to DAVID searching separately and only gene sets, where adj. p.value was lower than 0.05 were selected (Tab. 1). These two sets of genes was subjected to hierarchical clusterization procedure and presented as heatmap (Fig. 2).

In order to further explore known interaction between genes of interest, STRING-generated interaction network was generated among differentially expressed genes belonging to "fructose and mannose metabolism" KEGG pathway. Applied prediction methods that used textmining, co-expression, experimentally observed interactions (Fig. 3).

Discussion

Establishment and characterization of primary cell lines are crucial for application of these cells population and/or structurally advanced tissue as the grafts in regenerative medicine in both human and animals. Epithelial oviductal cell lines may be a valuable tool for elucidating many biochemical pathways existing in ovaries. In our study, we analyzed the transcriptome profile of porcine epithelial oviductal cells in different time periods after beginning of culture. Using a microarray approach, we demonstrated, for the first time, the role of the proteins involved in fructose and mannose metabolism during epithelial oviductal cells (OECs) long-term primary culture in vitro.

Heat map, showing obtained in this study results, indicates the variable mRNA expression profile of genes belonging to "fructose and mannose metabolism" KEGG pathway during long-term primary culture in vitro. From the whole transcript profile after microarray assay, we described 8 genes with variable transcripts expression profile during culture. According with our results we can distinguish few different patterns of mRNA expression. Two rate-limiting enzymes, HK2 and PFKFB3, playing pivotal

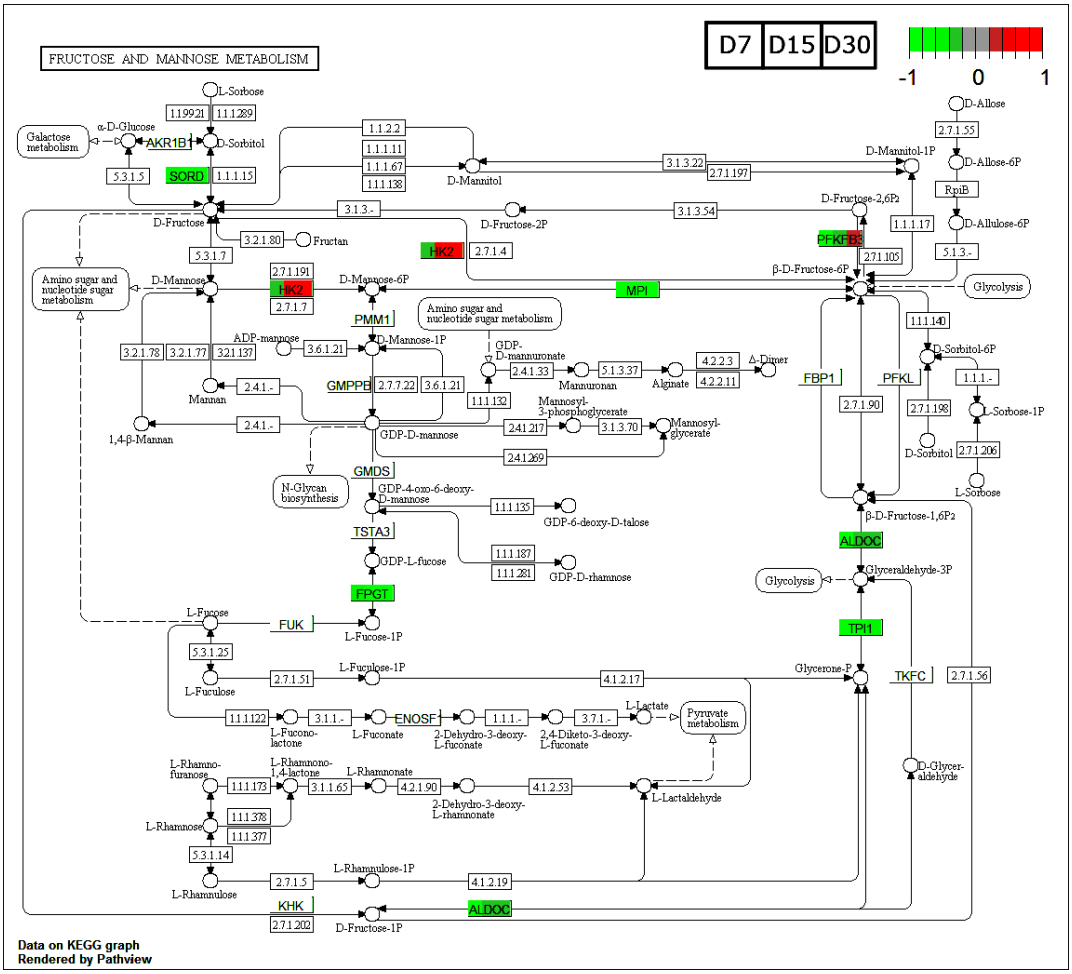

FIGURE 1 The "gluthatione metabolism" KEGG pathway with marked expression levels of differently expressed genes. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower expression). The boxes with names of genes were separated for three parts with representation of gene expression from the 7, 15 and 30 day of culture

TABLE 1 Fold changes, adjusted p values and ENTREZ gene ID of differentially expressed genes belonging to the "fructose and mannose metabolism" KEGG pathways. Symbols and names of the selected genes are also shown

Offical gene symbol	fold ratio 7D/24h	fold ratio 15D/24h	fold ratio 30D/24h	adj.p.value D7/24h	adj.p.value D15/24h	adj.p.value D30/24h	ENTREZ GENE ID
PFKFB3	1.098405	0.3201782	0.3190263	5.360659e-01	5.338184e-05	3.29722E-05	5209
PFKFB4	2.978245	3.6994733	2.5737602	1.317153e-04	3.366514e-05	1.219496e-04	5210
ALDOC	3.100575	1.2154949	1.3173597	3.006104e-04	2.745405e-01	1.104447e-01	230
FPGT	1.333621	1.6322642	2.0453385	2.290309e-01	4.297732e-02	6.752878e-03	8790
HK2	1.205102	0.3056736	0.2946793	5.615354e-01	1.810738e-03	1.175407e-03	3099
MPI	2.819985	1.9008635	1.8465988	9.246770e-05	6.754352e-04	6.272308e-04	4351
SORD	1.410967	1.5790706	2.7670722	7.039350e-02	2.148660e-02	3.030677e-04	6652
TPI1	2.751177	1.7373867	1.4210652	5.899703e-05	7.793195e-04	6.133121e-03	7167

role in carbohydrates metabolism, exhibit the first of the observed expression patterns. Both, HK2 and PFKFB3, indicate slightly increased transcript levels after 7 days of culture compare to start point (D1 on heat map) of our primary culture. Interestingly, during longer maintenance of the cell culture (after 15 and 30 days), we observed significant decrease in mRNA levels. HK2 and PFKFB3 transcript levels in OECs after 15 and 30 days of culture were lower even than in first day.

Hexokinase, primarily, catalyzes the first step in glucose metabolism, using ATP for the phospho-

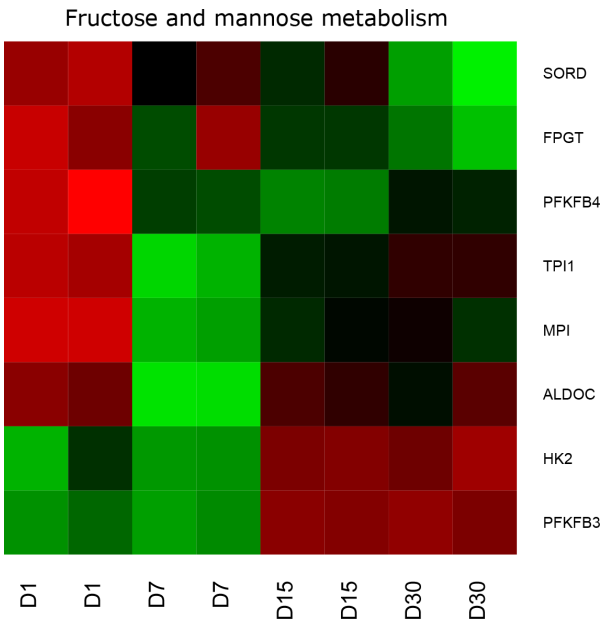

FIGURE 2 Heat map representation of differentially expressed genes belonging to the "fructose and mannose metabolism" KEGG pathway. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, lower expression). log2 signal intensity values were resized to Row Z-Score scale for any single genes (from -2, the lowest expression to +2, the highest expression)

rylation of glucose to glucose-6-phosphate (G6P). Four different types of hexokinase (HK1-4) were described in mammalian tissues. The two high affinity hexokinases, HK1 and HK2, play main role in the ATP dependent phosphorylation (HK3 is inhibited in physiological concentrations of glucose, whereas HK4, also known as glucokinase, is a low affinity hexokinase) [9-10]. However, while HK1 is ubiquitously expressed in the majority of adult tissues, HK2 is expressed at relatively high level only in adipose tissues, skeletal muscles, and heart [9]. This enzyme from first step of carbohydrates oxidation is also specific for mannose and fructose, thus HK2 can attach a phosphate residue to these hexoses. Despite its absence or low expression in the majority of adult normal cells, HK2 is expressed at high levels in many cancer cells. Jin and coworkers have shown significantly higher expression of HK2 in ovarian cancer tissues, especially in serous groups, than in normal ovarian [11]. These results highlight the importance of hexokinase 2 in maintenance active glycolysis even under aerobic conditions in malignant cells (Warburg effect). The high rate of glucose metabolism in cancer cells is being used to distinguish cancer cells from normal cells, thus HK2 expression differentiates cancer cells from normal cells. This conclusion has been con-

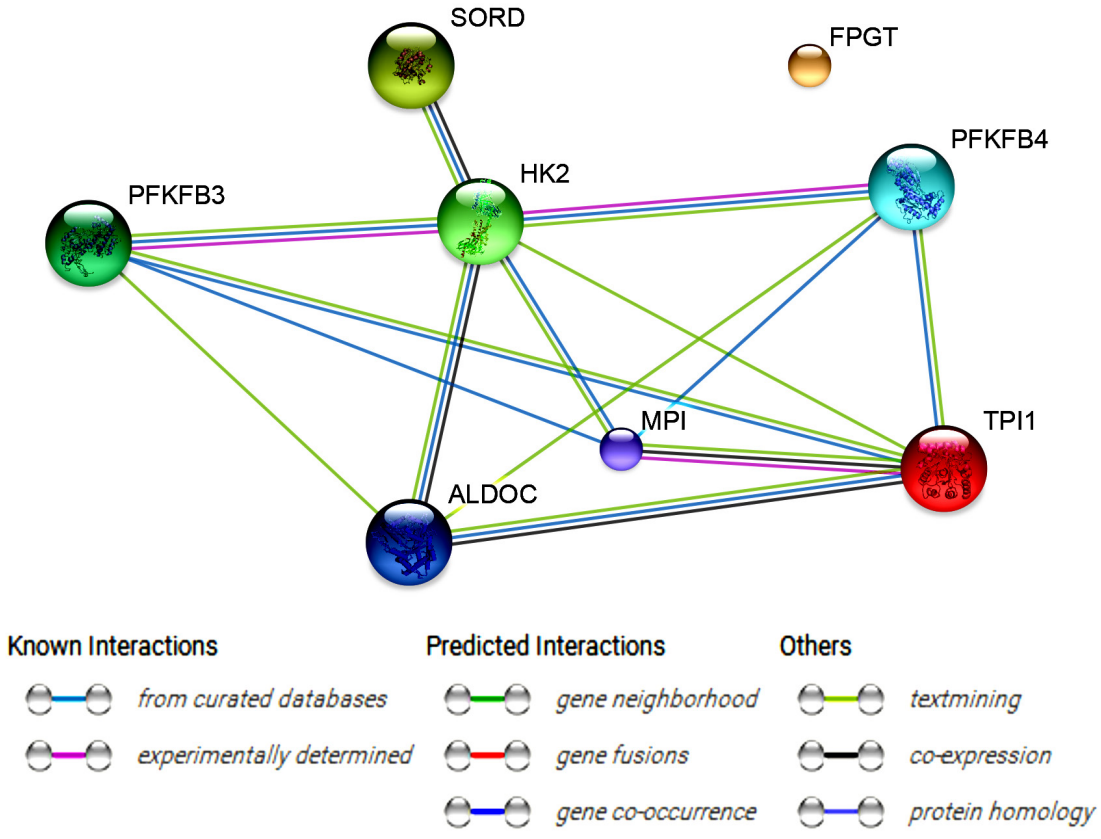

FIGURE 3 STRING-generated interaction network between differentially expressed genes, belonging to the "fructose and mannose metabolism" KEGG pathway. Applied prediction methods: text mining, co-expression, experimentally observed interactions

firmed in Patra and al. studies with mouse models of non-small cell lung cancer (NSCLC) and breast cancer [10]. Furthermore, in other studies investigators have demonstrated a relationship of HK2 overexpression with chemoresistance in epithelial ovarian cancer (EOC) [12]. The transcription factor Forkhead box protein M1 (FOXM1) play crucial role in aerobic glycolysis of EOC via activation of GLUT1 and HK2 transcription [13].

The bifunctional enzyme responsible for fructose-2,6-bisphosphate (F-2,6-P_2) synthesis was also described in our results, and exhibit similar transcript expression pattern to HK2. 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFKFB3, also known as PFK-2) via F-2,6-P_2 may promotes glycolysis. F-2,6-P_2 is the most potent positive allosteric effector of 6-phosphofructo-1-kinase (PFK-1), and hence of glycolysis [14]. Fructose and mannose metabolism pathways are linked with glucose oxidation via β-D-fructose-6P, obtained inter alia by the activity of PFKFB3. In cancer cells, PFK-2 is one of the factors, just like hexokinase, responsible for the Warburg effect [15]. Thus, over expression of PFKFB3 is observed in numerous cancers [16-17]. Other studies have shown that PFKFB3 regulates epithelial cells inflammatory status in response to macronutrients (glucose and palmitate intake) [18].

In our study, we observed the changing expression of other PFK-2 isoenzyme –PFKFB4 (all PFK-2 isoenzymes reviewed by Rider et al. [19]) during long-term cells culture. As detailed by Goren laboratory [20], expression of these isoenzymes is dependent on tissue and on development stage. Interestingly, our results indicate completely different mRNA expression pattern of PFKFB4 compare with PFKFB3. Transcript expression levels systematically increased in the early phase of primary cell culture (D7 and D15). Subsequently, after 30 days of culture, we observed decreased mRNA expression compare with previous measurements, nevertheless we obtained still definitely higher expression level than in start point (D1) of our cells culture. Therefore, we may postulate that PFKFB4 is the main enzyme which exhibit both kinase and bisphosphatase activity in OECs. In case of PFKFB3, we have shown decreased transcript expression during long-term culture (first of all measurement after 15 and 30 days of culture). According to these findings, we can say that PFKFB4 during longer periods of culture replaced by own activity the role of PFKFB3 which during culture systematically decreased.

Other profile of mRNA expression was shown by MPI, TPI1 and ALDOC. Here we can observe strong over expression after seven days of culture compare with first day. Then, we found decreased transcript expression levels after 15 and 30 days (excluding ALDOC which exhibit little higher mRNA level in 30D compare with 15D). Mannosephosphate isomerase (MPI) catalyzes the interconversion of fructose-6-phosphate and mannose-6-phosphate and plays a critical role in maintaining the supply of D-mannose derivatives. MPI mutations may impair protein glycosylation causing congenital disorder of glycosylation Ib (CDG-Ib) [21]. Other isomerase, triosephosphate isomerase 1 (TPI1) also was analyzed. It is involved in both glycolysis and gluconeogenesis, catalyzing the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate. Jiang and coworkers have suggested that TPI1 functioned as a tumor suppressor in hepatocellular carcinoma cells [22]. Vertebrates have 3 aldolase isozymes, aldolases A, B and C, which are distinguished by their catalytic properties. Aldolase C (ALDOC) is a brain type isozyme of a glycolysis enzyme [23]. Other authors characterized aldolase C as a hypoxia-regulated gene in epithelial cells from mouse lung [24]. However, to this day, there is no information about potential role of the described enzymes (MPI, TPI1 and ALDOC) in epithelial oviductal cells.

The last expression pattern contains genes (FPGT and SORD) with increased mRNA expression during whole culture (D7<D15<D30). Condensation of the β-L-fucose-1-phosphate with GTP to form GDP-beta-L-fucose is a metabolic function of fucose-1-phosphate guanylyltransferase (FPGT). While, sorbitol dehydrogenase (SORD) catalyzes the interconversion of polyols and their corresponding ketoses. In fructose and mannose metabolism pathway SORD is responsible for the creation of fructose using sorbitol as a substrate NAD(+) cofactor [25].

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Competing interests
The authors declare they have no conflict of interest

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