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GENES INVOLVED IN ANGIOGENESIS AND CIRCULATORY SYSTEM DEVELOPMENT ARE DIFFERENTIALLY EXPRESSED IN PORCINE EPITHELIAL OVIDUCTAL CELLS DURING LONG-TERM PRIMARY IN VITRO CULTURE – A TRANSCRIPTOMIC STUDY

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Abstract

An oviduct is an essential organ for gamete transport, oocyte maturation, fertilization, spermatozoon capacitation and early embryo development. The epithelium plays an important role in oviduct functioning. The products of secretory cells provide an optimal environment and influence gamete activities and embryonic development. The oviduct physiology changes during the female cycle, thus, the ratio of the secreted molecules in the oviduct fluid differs between phases. In this study, a differential gene expression in porcine oviduct epithelial cells was examined during the long-term primary in vitro culture. The microarray expression analysis revealed 2552 genes, 1537 of which were upregulated and 995 were downregulated after 7 days of culture, with subsequent changes in expression during 30 day-long culture. The obtained genes were classified into 8 GO BP terms, connected with angiogenesis and circulatory system development, extracted by DAVID software. Among all genes, 10 most up-regulated and 10 most down-regulated genes were selected for further investigation. Interactions between genes were indicated by STRING software and REACTOME FIViz application to the Cytoscape 3.6.0 software. Most of the genes belonged to more than one ontology group. Although studied genes are mostly responsible for angiogenesis and circulatory system development, they can also be found to be expressed in processes connected with fertilization and early embryo development. The latter function is focused on more, considering the fact that these genes were expressed in epithelial cells of the fallopian tube which is largely responsible for reproductive processes.

Running title: Upregulation of angiogenetic process in OEC primary cultures

Keywords: pig, oviducts, epithelial cells, microarray, long-term culture

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Introduction

The oviduct is an important organ of the female reproductive system. While the uterus plays a significant role in implantation, the oviduct is a place of gamete transport, oocyte maturation, fertilization, spermatozoon capacitation and early embryo development [1,2]. In most mammals, the oviduct consists of three different segments, the infundibulum, ampulla, and isthmus, each of which is involved in specific biological events.

The mammalian oviduct is lined by a simple columnar epithelium [3]. Epithelial cells are formed in the early stage of embryo development and are generated from ectoderm and endoderm. The function of epithelial cells is to transport essential nutrients, oxygen, fluids, and ions. They also secrete several components and produce certain signals. These signals might be generated spontaneously or due to other factors [4]. The epithelium of the oviduct plays an important role in the functioning of this structure. It provides a proper environment for the embryonic development at its early stage [5]. It is composed of two different porcine oviduct epithelial cell (POEC) types, ciliated cells and non-ciliated secretory cells. These two types of cells can be easily differentiated in pigs, based on the differences observed in the cytoplasmic concentration of the cells, the secretory granules found on the apical surface of the cells, and the presence or lack of cilia [1]. Secretory cells synthesize and secrete specific proteins, glycoproteins and glycosaminoglycans (GAGs) that influence the activities of gametes and embryos at the cleavage-stage. The synthesized secretory material is stored in the epithelial cells to later be released into the lumen of the fallopian tube [1,6].

Oviduct fluid is an essential part of oviductal environment. It consists of serum filtrate, follicular fluid and specific secretory compounds that are synthesized and secreted by the epithelial secretory cells [7]. A composition of the oviductal fluid changes during the female cycle, due to hormonal changes in the oviduct [8,9]. The oviductal physiology alters in a cyclical manner, modulated by steroid hormones [10]. At the follicular phase of the estrous cycle, porcine oviductal epithelium contains increased number of long ciliated cells, and the secretory function of epithelial cells is higher due to estrogen and LH stimulation. However, during the luteal phase, numerous spherical-shaped, non-ciliated cells with short microvilli are present on the apical surface [7,11]. These alterations are necessary to provide an optimal conditions for gamete functioning, fertilization and early embryonic development [12].

Pig serves as a favored model organism due to its close anatomical and physiological similarities to human. Porcine female reproductive biology, including the hormonal regulation during the estrus cycle and early events of reproduction, resembles characteristics of human female reproductive system. The possibility to apply the results in human physiology, and easy availability, make pigs one of the most interesting animal models [13]. It is important to note that some specific features and functions of the oviductal epithelium may be lost during *in vitro* culture.

This study investigated differential gene expression in the porcine oviductal epithelial cells during 30 days of culture. The obtained genes were classified into 8 different ontology groups connected with angiogenesis and circulatory system development. However, most of them also exhibit other functions in different organs or are strictly connected with early embryonic development.

Materials and Methods

Animals

In this study, crossbred gilts (n=45) at the age of about nine months, 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 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. The cell suspension was centrifuged (200 x g, 10 min.). Next, the cells were washed in PBS and centrifuged again. 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. The culture lasted 30 days.

RNA extraction from oviductal epithelial cells (OECs)

Oviductal epithelial cell were pooled and harvested at 24h, 7 days, 15 days and 30 days after the 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, 100 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 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 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 Multi-array 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 by examination of genes involved in angiogenesis and circulatory system development. The differentially expressed gene list was uploaded to the DAVID software (Database for Annotation, Visualization and Integrated Discovery) [14]. Subsequently the relation between the genes belonging to chosen GO terms with GOplot package was analyzed [15]. The GoPlot package had 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. This information allowed for estimating the change course of each gene-ontology term.

Moreover, interactions between differentially expressed genes/proteins belonging to the chosen GO terms were investigated by the STRING10 software (Search Tool for the Retrieval of Interacting Genes) [16]. 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.

Finally the functional interactions between genes that belongs to the chosen GO BP terms were investigated by REACTOME FIViz application to the Cytoscape 3.6.0 software. The ReactomeFIViz app is designed to find pathways and network patterns related to cancer and other types of diseases. This app accesses the pathways stored in the Reactome database, allowing to do 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 app 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. 83/2012/DNT.

Results

To investigate oocyte transcriptome changes following 7, 15 and 30 days after start of primary porcine oviductal epithelial cells, whole gene expression analysis by Affymetrix® Porcine Gene 1.1 ST Array was performed. 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 that group that consists of 2552 different genes, 1537 were up-regulated and 995 were down-regulated after 7 days of culture, 1471 were up-regulated and 1061 were down-regulated after

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

	Gene Symbol	foldD7_D1	foldD15_ D1	foldD30_ D1	adj.P.Val. D1_D7	adj.P.Val. D1_D15	adj.P.Val. D1_D30	Gene Id
8720	CELA1	0,10027	0,111224	0,091977	4,67E-05	3,35E-05	1,41E-05	396766
11378	ANPEP	0,143464	0,201711	0,482675	1,74E-05	1,96E-05	0,000794	397520
7934	SNAI2	0,183766	0,134521	0,130079	0,000411	0,000129	7,85E-05	641345
11998	CXCL10	0,241713	0,447019	0,331634	0,002165	0,025903	0,004916	494019
1636	ARMC4	0,265889	0,233069	0,203708	0,00012	4,77E-05	1,94E-05	1,01E+08
7176	HBB	0,267391	0,278031	0,272145	0,00381	0,003686	0,002679	1,01E+08
9148	ERBB3	0,270208	0,12388	0,117998	4,67E-05	3,53E-06	1,48E-06	---
2546	KDM6B	0,300172	0,278756	0,296044	0,0001	4,81E-05	3,97E-05	---
4343	ITGAV	5,814753	6,506911	6,737856	0,000126	6,01E-05	3,42E-05	397285
7340	EPAS1	7,629974	6,655106	16,82792	8,57E-06	5,17E-06	5,84E-07	1E+08
4653	FN1	8,29918	15,9846	14,15968	4,67E-05	7,40E-06	5,68E-06	397620
2727	PPARD	9,200973	6,826522	5,602991	2,25E-05	2,24E-05	2,49E-05	397671
3603	HHEX	9,447447	11,82395	11,30739	2,69E-05	9,59E-06	6,25E-06	397232
12220	NOX4	11,05623	21,12763	32,07053	4,90E-06	9,73E-07	2,31E-07	1,01E+08
2314	ITGB3	12,67365	13,15962	10,9347	4,27E-06	1,55E-06	8,03E-07	397063
12376	COL1A2	15,57291	56,37555	61,32906	3,63E-06	7,45E-07	2,31E-07	1,01E+08
6887	LOX	29,0517	43,84613	46,65297	7,88E-06	2,86E-06	1,06E-06	1,01E+08
12730	THY1	38,75283	32,74287	31,30186	3,01E-06	1,39E-06	5,59E-07	1E+08

15 days of culture and 1329 were up-regulated and 1203 were down-regulated after 30 days of culture.

Among these, genes belonging to "angiogenesis", "blood circulation", "blood vessel development", "blood vessel morphogenesis", "circulatory system development" , "regulation of heart contraction", "regulation of heart rate" and "vasculature development" Gene Ontology Biological Process (GO BP) terms were extracted by the DAVID (Database for Annotation, Visualization and Integrated Discovery) software. Up- and down-regulated genes included in selected GO BP terms were presented, together with their fold changes and adj. p values, in **table 1**. All eight sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (**Fig. 1**). To investigate the direction of changes between each selected GO BP term, its z-score (the ratio of up- and down-regulated genes in each GO BP terms) was calculated. The z-scores with the representation of up- and down-regulated genes were shown in series of circle diagrams (**Fig. 2**).

For further analysis 10 most up- and 10 most down-regulated genes were chosen from 91 genes that built chosen GO BP terms.

In Gene Ontology database, genes that formed one particular GO group 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 heatmap (**Fig. 4**).

STRING software was used to generate interaction network among differentially expressed genes belonging to each of selected GO BP terms. This prediction method provided us 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 **figure 6**.

Discussion

Genes isolated from the oviductal epithelial cells were classified into 8 different ontology groups: "angiogenesis", "blood vessels development", "blood vessels morphogenesis", "circulatory system development", "blood circulation", "vasculature development", "regulation of heart rate" and "regulation of heart contraction". These groups include genes that are generally responsible for development and regulation of cardiovascular system. From 2552 genes, we chose 10 most up-regulated and 10 most down-regulated genes which represent groups mentioned above, as well as other groups, connected with different organs and early embryo development. We investigated the expression of these genes for 30 days, checking the differences after 7, 15 and 30 days. All genes constantly changed their expression.

The most up-regulated gene was *THY-1* (*T-cell surface antigen*) which is expressed not only on immune cells, but also non-immune cells, such as endothelial cells, mesenchymal stem cells, some fibroblasts and neurons [17]. It is e.g. responsible for development of myelin sheath, surrounding axons

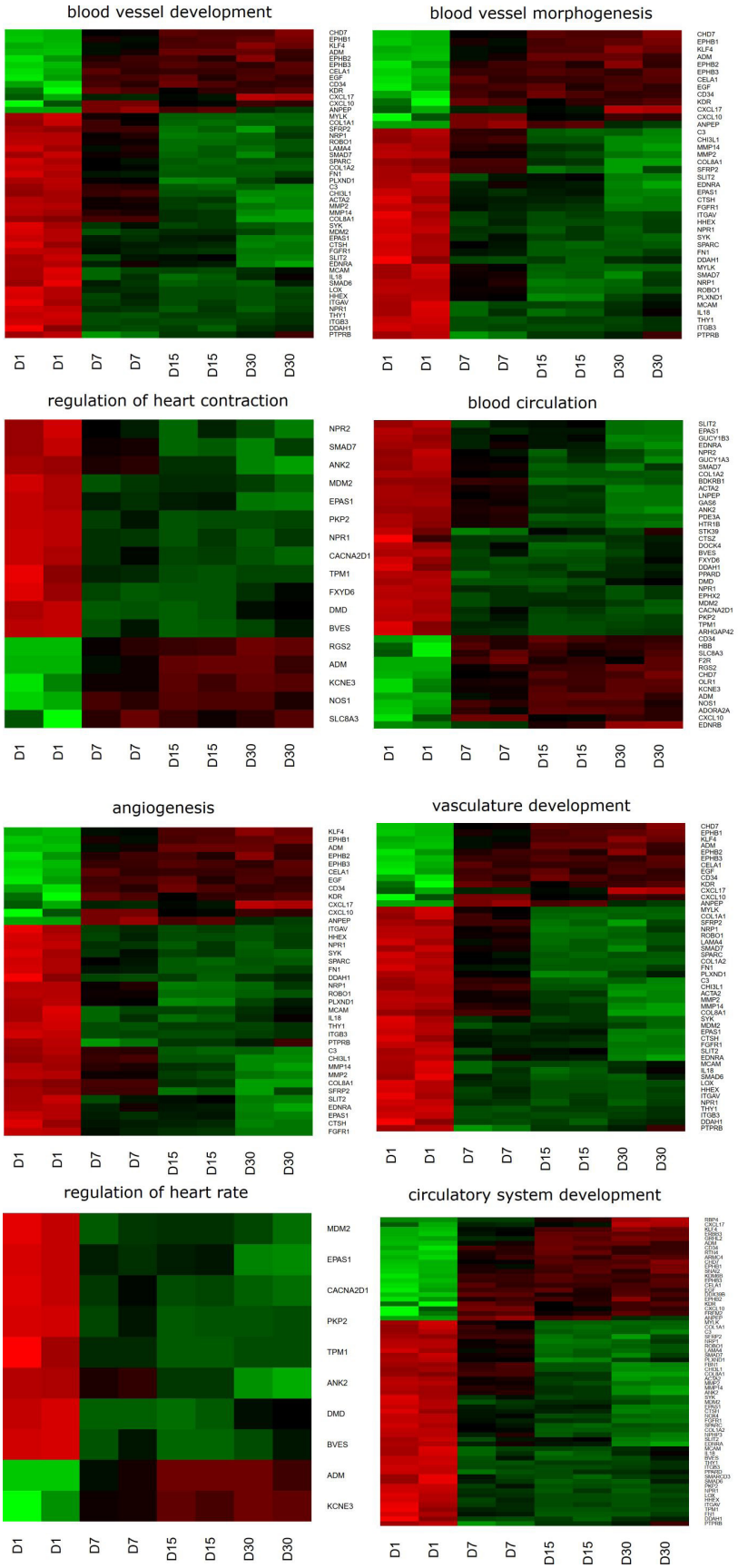

FIGURE 1 Heatmap representation of differentially expressed genes belonging to the "angiogenesis", "blood circulation", "blood vessel development", "blood vessel morphogenesis", "circulatory system development" , "regulation of heart contraction", "regulation of heart rate" and "vasculature development" GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colors (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)

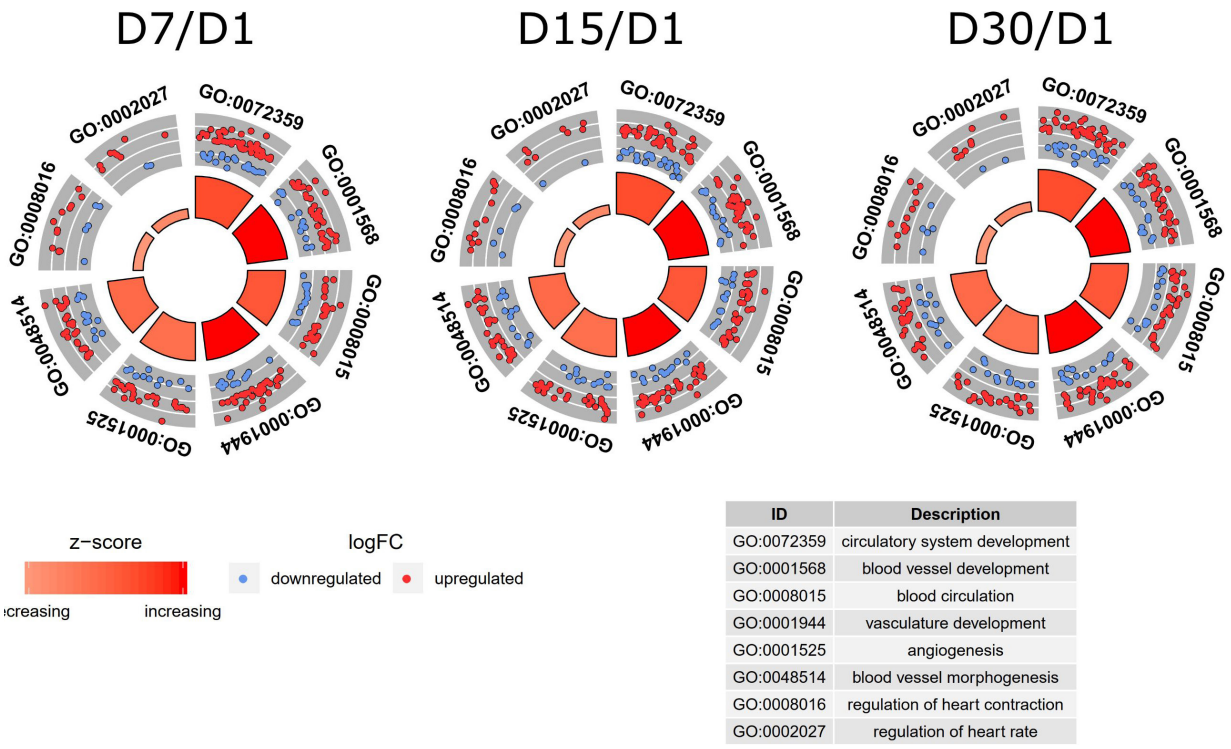

FIGURE 2 The circle plots showing the differently expressed genes and z-score of the studied GO BP terms. The outer circle of each plot shows a scatter plot for each term of the fold change of the assigned genes. Red dots display up-regulation and blue ones down-regulation. The inner circle shows the 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

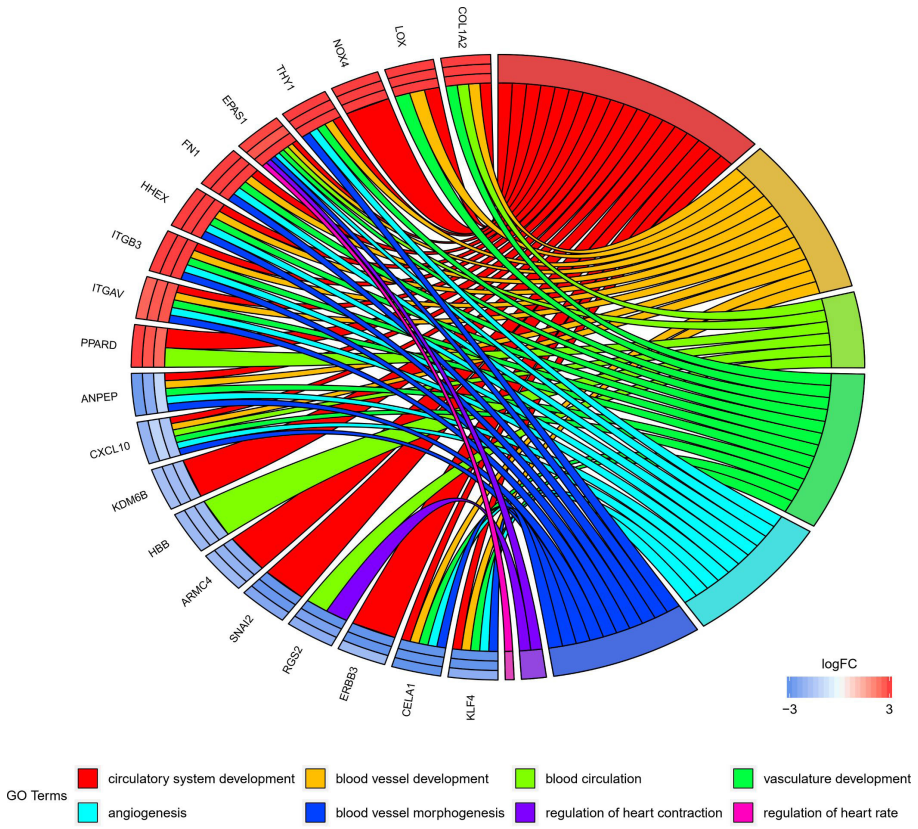

FIGURE 3 The representation of the mutual relationship between 10 most up and down regulated genes that belongs to studied GO BP terms. The ribbons indicate which gene belongs to which categories. The genes were sorted by logarithm of Fold Change (logFC) from most to least changed gene. The bars represent are placed between ribbons and gene symbols. The outer bars represent logFC between 7 and 1 day, middle between 15 and 1 day and inner between 30 and 1 day of cell culture

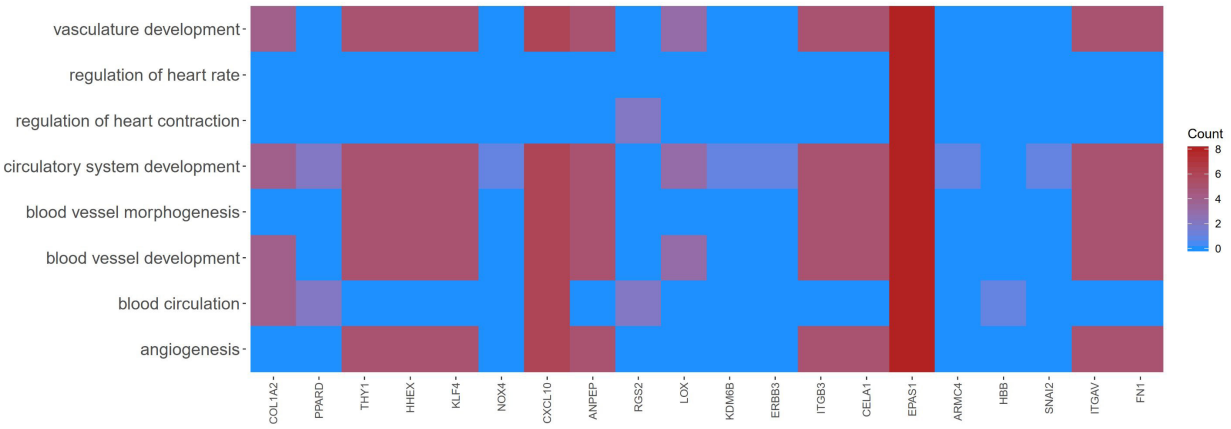

FIGURE 4 Heatmap showing the gene occurrence between 10 most up and down regulated genes that belongs to studied GO BP terms. the coloring of the tiles represents each gene depends on the presence or absence of gene in listed GO BP term. The intensity of the color grows with the number of GO BP terms in which given gene co-occur

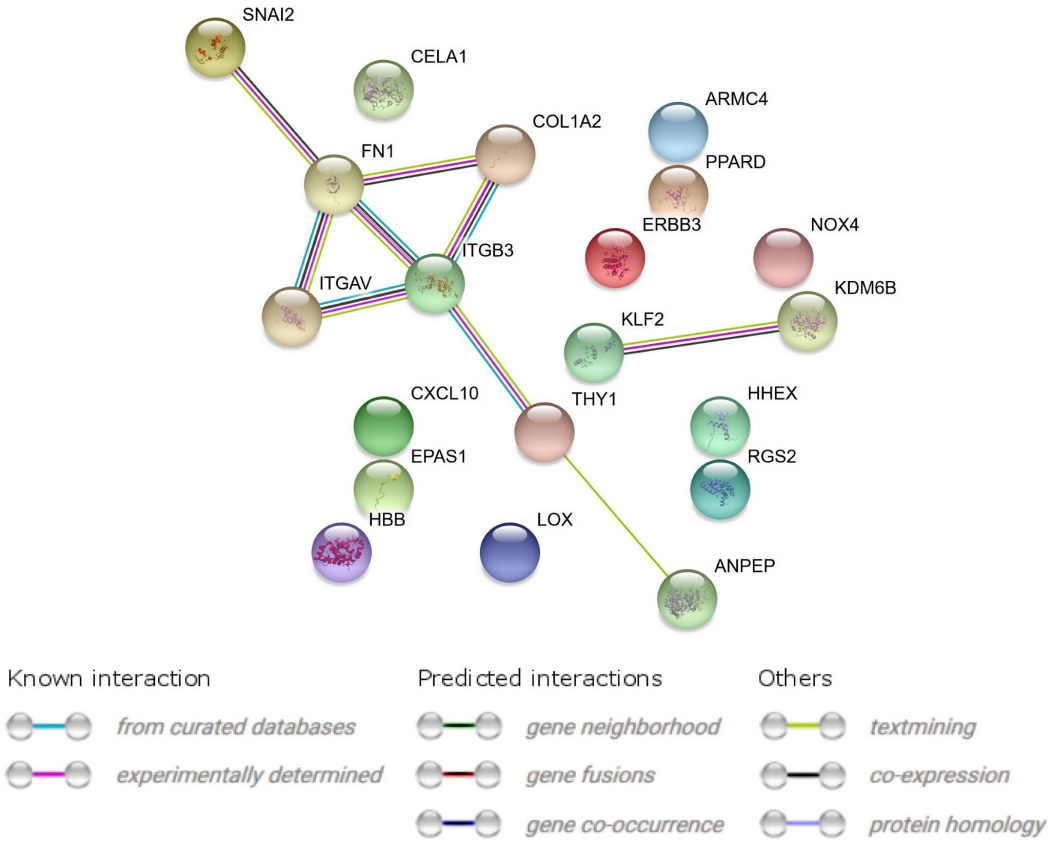

FIGURE 5 STRING-generated interaction network among 10 most up and down regulated genes that belongs to studied GO BP terms. The intensity of the edges reflects the strength of interaction score

of neurons of the nervous system. Its significant role in nervous tissue development may be of relevance to disorders such as ataxia-telangiectasia that combines neurologic and immunologic defects [18]. In earlier research performed on rats, *THY-1* was identified on the endothelium of newly formed blood vessels. However it was expressed only during adult angiogenesis, not in embryos [19].

It appears that most of up-regulated genes from oviductal epithelium are closely related with early embryonic development. *PPARD* (*peroxisome proliferator-activated receptor delta*) has been found in the "embryo implantation", "multicellular organism development" and "placenta development" ontology groups. In the latter it is responsible for decidualization, the process in which the cellular and vascular changes occur in the endometrium of uterus just after the implantation of blastocyst. Besides, *PPARD* is a major regulator of lipid metabolism and can be activated by either fatty acids or their metabolic derivatives [20].

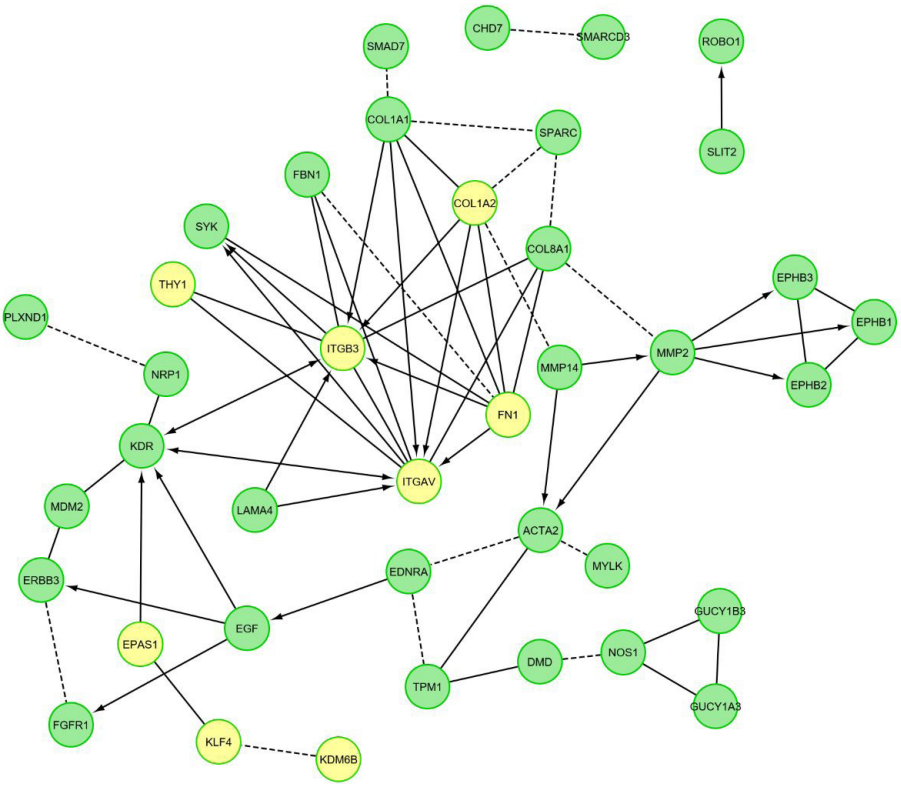

FIGURE 6 Functional interaction (FI) between differentially expressed genes belonging to the selected GO BP terms. In following figure "->" stands for activating/catalyzing, "-|" for inhibition, "-" for FIs extracted from complexes or inputs, and "---" for predicted FIs. The 10 most up and down regulated genes were marked yellow. The missing genes do not appear in reactome database

The next gene is *ITGAV* (*integrin, alpha-v*), which also belongs to "embryo implantation" ontology group. Integrins are key receptors for several cellular processes including cytoskeletal organization, cell proliferation and differentiation. Alpha-V integrins participate in many developmental processes and can be therapeutic targets for inhibition of angiogenesis and osteoporosis [21]. Other study indicated that mice lacking *ITGAV* gene in endothelial and hematopoietic cells died from vascular and developmental defects [22].

Another gene found in "embryonic placenta development" ontology group is *EPAS1* (*endothelial PAS domain protein-1*). In adult humans it is usually expressed in highly vascularized tissues and in mice it is expressed in both adult and embryonic endothelial cells. It has been observed that *EPAS1* may play a role of a vascularization regulator, including regulation of gene expression in the endothelial cells in response to hypoxia [23]. Since placenta is an organ highly involved in oxygen exchange, the relatively high expression of EPAS1 may be connected with response to hypoxia [24]. Additionally EPAS1 is included in „blood vessel remodeling" ontology group.

HHEX (*haematopoietically expressed homeobox*) encodes a homeobox transcription factor, which may be involved in hematopoietic differentiation and, as many members of this family, in developmental processes. It hasn't been found in placenta, however, according to NEXTPROT database, this gene is responsible for "in utero embryonic development" in mice, the process of the embryo progression in the uterus, from zygote formation in the oviduct to the time of birth. Foley and Mercola [25] observed the *HHEX's* significance in fetal cardiac development. Paz et al. [26] indicates that knockout of this gene in mice results in embryonic lethality.

Earlier mentioned *ITGAV* interacts and is co-expressed with *FN1* (*Fibronectin-1*)(Fig.5). Its expression is increased in oviductal epithelial cells around ovulatory period [27], due to its role in regulation of oviduct cell-sperm interaction, fertilized oocyte activation and embryo development. Fibronectin domains interact with integrins and bind to oviductal cells and extracellular molecules, which is essential in cell adhesion, migration, proliferation and differentiation processes. Both fibronectin and integrins participate e.g. in oocyte uptake by oviduct fimbriae [28] and embryo development [29]. Besides, it has been found in the research on zebrafish that fibronectin is required for early cardiomyocyte formation in heart development [30].

ITGB3 (*Integrin beta-3*) is another integrin strongly interacting with fibronectin. It is involved in implantation processes, promotes intracellular adhesion and influences the uterine-conceptus in-

teractions as the receptor of ECM proteins [31]. It has been found in the endometrium and the embryo during implantation events in several species including human [31,32]. Both integrin subunits – aV and b3 have been observed to be expressed in early embryo implantation in pigs [33]. The importance of integrins in implantation was proved by Illera et al. [34] in studies on mice, where blocking of aVb3 integrin expression inhibited embryo implantation. In turn, the alterations in integrin expression in endometrium caused infertility in humans [35]. Moreover, *ITGB3* is responsible for maintaining capillary integrity and plays an essential role in regulating vascular permeability [36].

NOX4 (*NADPH oxidase 4*), is an up-regulated gene which was classified to only one of 8 ontology groups in our research – "circulatory system development". Its major function is catalyzing ROS formation. It is expressed in kidney tubular cells at high levels and at lower levels in cardiomyocytes, endothelial cells and other cell types [37], what explains relatively low expression of this gene in our study. It has been shown that reactive oxygen species are significant in mediation of angiogenesis. *NOX4* producing H_2O_2 in endothelial cells directly leads to angiogenesis [38]. Additionally it is classified to "cardiac muscle cell differentiation" and "heart process" ontology groups which are strictly connected with circulatory system development.

COL1A2 (*collagen type I alpha 2 chain*) is a gene responsible mostly for skeletal system development. Collagen type I is predominantly produced by mesenchymal cells in a tightly controlled manner. Its structure and biosynthesis are crucial for growth, morphogenesis and tissue repair [39]. Mutation in *COL1A2* gene causes osteogenesis imperfecta (OI), a disorder of connective tissue, where bones are fragile and susceptible to fracture [40]. Knisely et al. [41] observed that *COL1A2* gene mutation in an infant contributed to death at 22 days of age, due to complications of osteogenesis imperfecta. *COL1A2* interacts with integrins which act as receptors binding collagen. Although our research doesn't indicate it, according to literature *COL1A2* also interacts with another up-regulated gene – *LOX* (*lysyl oxidase*). *LOX* mediates collagen fiber organization in the ECM which is important in osteoblastogenesis regulation [42]. The aberrations in collagen type I cross-linking have been observed in bone disorders including osteopetrosis or osteoporosis [43,44]. *LOX* modulates cross-linking of collagen monomers, forming insoluble fibers in the ECM, thus, it plays a significant role in maintaining the connective tissue integrity [42].

Among 10 genes down-regulated in *in vitro* conditions, *CELA1* (*chymotrypsin like elastase family member 1*) was the most down-regulated. *CELA1* is known to be expressed in porcine pancreas but in human pancreas it remains functionally silent [45]. According to Liu et al. [46] research, *CELA1* was observed to regulate tubule formation in an *in vitro* angiogenesis assay. This may suggest that it is responsible for forming tubular structures such as blood vessels in *in vivo* conditions as well.

ANPEP (*alanyl aminopeptidase*) is a potent angiogenic regulator. Petrovic et al. [47] showed that this gene was required for endothelial cell membrane integrity and protein organization. Rangel et al. [48] observed that *ANPEP* knockout mice undergo physiologic angiogenesis. However under pathologic conditions, their angiogenic response is seriously impaired. *ANPEP* is considered to interact with *THY-1* (Fig.5).

CXCL10 (*C-X-C motif chemokine 10*), like most chemokines, plays a significant role in development and homeostasis. It influences central nervous system and endothelial cells which are involved e.g. in angiogenesis. It has been showed that *CXCL10* is a strong inhibitor of angiogenesis *in vivo*. Potentially, it participates in angiogenesis regulation during inflammation and tumorigenesis [49].

Among the chosen down-regulated genes, 5 were classified to only one ontology group. *SNAI2, ARMC4, ERBB3* and *KDM6B* were found in "circulatory system development" group, and *HBB* was found in "blood circulation" group. *HBB* (*Hemoglobin subunit beta*), along with *HBA* (*alpha globin*), determines a structure of adult hemoglobin and is included in "blood circulation" ontology group, due to important role of hemoglobin in supplying tissues with oxygen. Mutations in this gene may lead to diseases including erythrocytosis, beta-thalassemia and sickle cell anemia. Interestingly, some *HBB* mutations display also beneficial features such as resistance to malaria [50]. *SNAI2* (*snail family transcriptional repressor 2*) plays a crucial role in developmental processes and is especially responsible for epithelium development and epithelial-mesenchymal transitions [51]. In embryonic life, it is one of the genes which control differentiation and migration processes of neural crest cells. It has been noted that in humans it exhibits antiapoptotic activity in both developing embryo and adults, preventing productive cell death [52]. *ERBB3* (*erb-b2 receptor tyrosine kinase 3*) plays important role in nervous system development and muscle tissue development. It has been observed that most embryos with *ERBB3* knockout die before birth due to lack of Schwann cells that normally co-occur with peripheral axons of neurons [53]. Makinodan et al. [54] showed on mice that social isolation immediately after weaning caused reduced expression of *ERBB3* leading to irreversible alterations in myelination and function of prefrontal cortex. *KDM6B* (*lysine-specific demethylase 6B*) was observed to have increased expression in murine embryonic stem cells during endothelial cell differentiation [55]. Agger et al. [56] found that mutation or inhibition of this gene leads to abnormal gonad development. Thus, its proper functioning is important for normal embryo development. *KDM6B*

was also found to play a key role in posterior development through HOX gene expression regulation [57].

KDM6B and *KLF4* (*Kruppel-like factor 4*) are predicted to interact with each other (Fig.6). It is possible due to the potential role of *KLF4* in embryonic stem cells. However, while *KDM6B* has increased expression during ES cells differentiation, the *KLF4* is highly up-regulated in undifferentiated ES cells, and during differentiation its expression dramatically decreases [58]. Thus, *KLF4* plays a key role in ES cell maintenance and prevents their differentiation. *KLF4* was classified e.g. to "negative regulation of angiogenesis" group which is related with prevented differentiation of ES cells into endothelial cells. *KLF4* was observed to strongly inhibit the endothelial cells and vascular smooth muscle cells proliferation. It also suppresses the potential of endothelial cells to form capillary-like structures. *KLF4* indirectly causes anti-proliferative and anti-angiogenic effects in ECs and VSMCs. Zheng et al. [59] suggests that enhancing *KLF4* expression might be a potential strategy in treatment of pathological vascularization and proliferative vascular disorders.

RGS2 (*Regulator of g protein signaling 2*) is additionally classified into "relaxation of cardiac muscle" and "relaxation of vascular smooth muscle" ontology groups. Tang et al. [60] observed that *RGS2* knockout mice exhibit serious hypertension and enhanced contractions of blood vessels. Heximer et al. [61] concluded that alterations in the *RGS2* function or expression may be a cause of hypertension development in humans. Thus, it appears that *RGS2* is necessary for proper functioning of vascular system and normal blood pressure and, due to its function, may be considered as a new hypertension drug development target [60].

Our current results showed differential expression of genes during long-term culture, confirming that *in vitro* conditions are significantly different from those *in vivo*. The microarray analysis showed that genes expressed in the oviductal epithelial cells can display other functions that are not strictly connected with oviduct function. However, circulatory system development and angiogenesis are closely related with early embryo development and other events occurring in oviductal lumen.

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

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