



ONTOLOGY GROUPS REPRESENTING ANGIOGENESIS AND BLOOD VESSELS DEVELOPMENT ARE HIGHLY UP-REGULATED DURING PORCINE OVIDUCTAL EPITHELIAL CELLS LONG-TERM REAL-TIME PROLIFERATION – A PRIMARY CELL CULTURE APPROACH

Mariusz J. Nawrocki¹, Piotr Celichowski², Maurycy Jankowski¹, Wiesława Kranc¹, Artur Bryja¹, Sylwia Borys-Wójcik¹, Michał Jeseta³, Paweł Antosik⁴, Dorota Bukowska⁴, Małgorzata Bruska¹, Maciej Zabel^{5,6}, Michał Nowicki², Bartosz Kempisty^{1,2,3}

Abstract

The morphological and biochemical modification of oviductal epithelial cells (OECs) belongs to the group of compound processes responsible for proper oocyte transport and successful fertilization. The cellular interactions between cumulus-oocyte complexes (COCs) and oviductal epithelial cells (OECs) are crucial for this unique mechanism. In the present study we have analyzed angiogenesis and blood vessel development processes at transcript levels. By employing microarrays, four ontological groups associated with these mechanisms have been described. Differentially expressed genes belonging to the “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms were investigated as a potential markers for the creation of new blood vessels in cells under in vitro primary culture conditions.

Running title: Regulation of oviductal blood vessels morphogenesis

Keywords: pig, oviducts, epithelial cells, microarray, in vitro primary culture

¹Department of Anatomy, Poznan University of Medical Sciences, Poznan, Poland

²Department of Histology and Embryology, Poznan University of Medical Sciences, Poznan, Poland

³Department of Obstetrics and Gynecology, University Hospital and Masaryk University, Brno, Czech Republic

⁴Veterinary Center, Nicolaus Copernicus University in Torun, Torun, Poland

⁵Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland

⁶Division of Anatomy and Histology, University of Zielona Góra, Zielona Góra, Poland

* **Correspondence:** bkempisty@ump.edu.pl

Full list of author information is available at the end of article

Introduction

The histological architecture of oviducts undergoes substantial modification in response to the female reproductive cycle. These morphological modifications are substantially accompanied by changes within ovary and ovarian follicles during ovulation. The morphological changes are accompanied by changes in cellular metabolism, which is associated with secretion of hormones, and/or synthesis of growth factors, that facilitate the transport of fully mature MII oocytes through the oviduct before fertilization. The cellular interactions between cumulus-oocyte complexes (COCs) and oviductal epithelial cells (OECs) are crucial for this unique „journey“. Moreover, the tissue and cell plasticity appear to be important factors determining proper gamete localization, and are crucial for successful monospermic fertilization. Therefore, it has been suggested that the normal fertilization is a compound process regulated by proper oviductal receptivity and sensitivity as well as subsequent recognition, interaction and fusion of the gametes.

Our recent experiments indicated that the gene expression profile changes within the OECs were also accompanied by significant changes in their *in vitro* proliferation capability. The long-term *in vitro* cultivation (IVC) demonstrated an increase of Cell Proliferation Index after 48-72 hours, which was associated with changes in transcriptomic profile. The progress of OECs' *in vitro* proliferation may be orchestrated by their biochemical and metabolomic profile, which is directly associated with cellular senescence. However, the increased *in vitro* proliferation ability of the OECs was also accompanied by a differentiation capability, which may be recognized as the „fingerprint“ of their developmental competence and stemness specificity during long-term *in vitro* primary culture.

The angiogenesis and blood vessel development are compound processes, directly associated with organogenesis and tissue regeneration. One of the biggest clinical concepts of the 21st century are artificial organs formation, 3D bio-printing and regenerative and reconstructive medicine. The formation of new vessels that supply a tissue during regeneration is crucial for tissue reconstruction.

It is suggested that the blood vessels development and neoangiogenesis are important factors determining tissue specific reorganization and cellular morphological modification within the reproductive tract, especially in the oviduct and endometrium. However, the molecular basics of these processes in porcine oviducts was never investigated before. Moreover, this study aimed to investigate the transcriptomic profile of genes regulating angiogenesis and blood vessel development in OECs, in conditions of long term *in vitro* culture.

Material 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 Na₂HPO₄, 2 mM KH₂PO₄, 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 1h 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 cells were centrifuged (200 g, 10 min.), washed in PBS and centrifuged again. Then, the cells 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 were 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% CO₂. Once the OEC cultures attained 70–80% confluency, they were washed with PBS and passaged. The passage procedure involves cell digestion with 0.025% Trypsin/EDTA, enzyme neutralization by a 0.0125% trypsin inhibitor (Cascade Biologics, Portland, USA), centrifugation of samples, and resuspension at a seeding density of 2x10⁴ cells/cm². The culture medium was changed every three 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 *in vitro* the 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 1000 spectrophotometer, Thermo Scientific). The RNA integrity and quality were checked on a Bio-

analyzer 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/ μ l 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 μ g) 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, the Robust Multiarray Averaging (RMA) algorithm was used. 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 the GO processes of interest. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery) [1], where genes belonging to "angiogenesis", "blood circulation", "blood vessel development" and "blood vessel morphogenesis" GO BP terms were extracted. Expression data of these genes were also subjected to a hierarchical clusterization procedure, and their expression values were presented as a heat map.

Subsequently we analyzed the relation between the genes belonging to chosen GO terms with GOplot package [2]. The GoPlot package had calculated the z-score: the number of up-regulated genes mi-

nus 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.

Interactions between differentially expressed genes/proteins belonging to the studied gene ontology group were investigated by the STRING10 software (Search Tool for the Retrieval of Interacting Genes) [3]. 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 Reactome FIViz 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

Whole transcriptome profiling by Affymetrix microarray allow 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 consists of 2533 different transcripts.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of gene ontology biological process terms (GO BP) that contain differently expressed transcripts. Up and down regulated gene sets were subjected to DAVID search separately, and only gene sets where adj. p value were lower than 0.05 were selected. The DAVID software analysis showed that diffe-

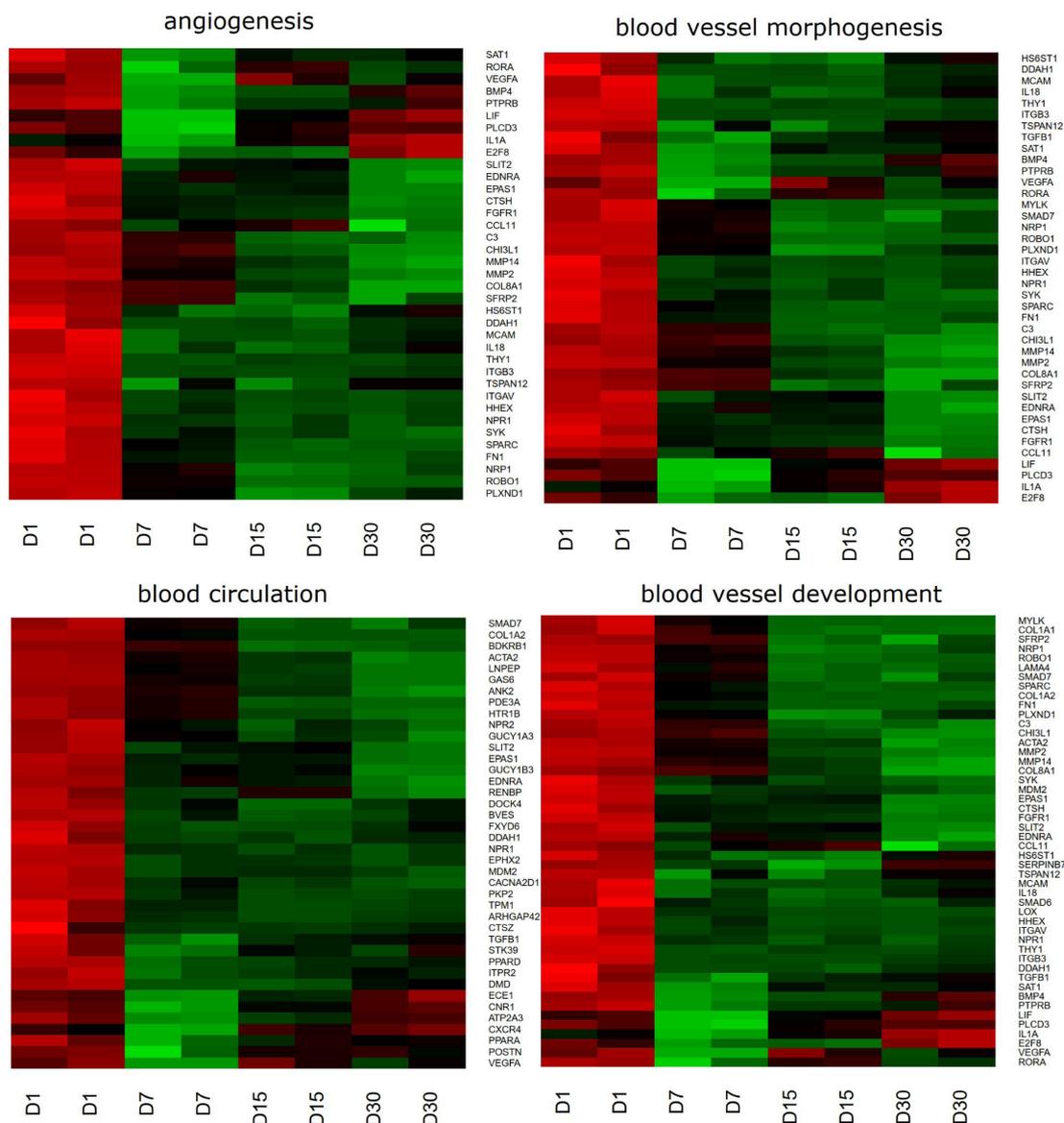


FIGURE 1 Heat map representation of differentially expressed genes belonging to the “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, 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)

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

Gene symbol	Fold. Change D15/D7	Fold. Change D30/D7	Fold. Change D30/D15	adjusted P.Value D15/D7	adjusted P.Value D30/D7	adjusted P.Value D30/D15
DDAH1	2.193807786	2.237610226	2.002712688	0.001252407	0.000855389	0.001404956
EDNRA	2.273637629	2.466966484	4.26265854	0.000753134	0.000360677	2.11E-05
NRP1	2.914640843	7.413916735	5.602078722	0.000288975	9.31E-06	1.15E-05
VEGFA	4.216230322	1.241897384	2.156261928	0.000763234	0.451948554	0.010972277
SLIT2	4.503980958	3.402733417	7.662300696	0.00011686	0.000211038	1.00E-05
EPAS1	7.62997386	6.655105812	16.82792308	8.57E-06	5.17E-06	5.84E-07

rently expressed genes belongs to 657 Gene ontology terms. In this paper we focused on 72 genes that belong to “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphoge-

nesis” GO BP terms, all of which were upregulated. These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (Fig. 1). The gene symbols, fold changes in expres-

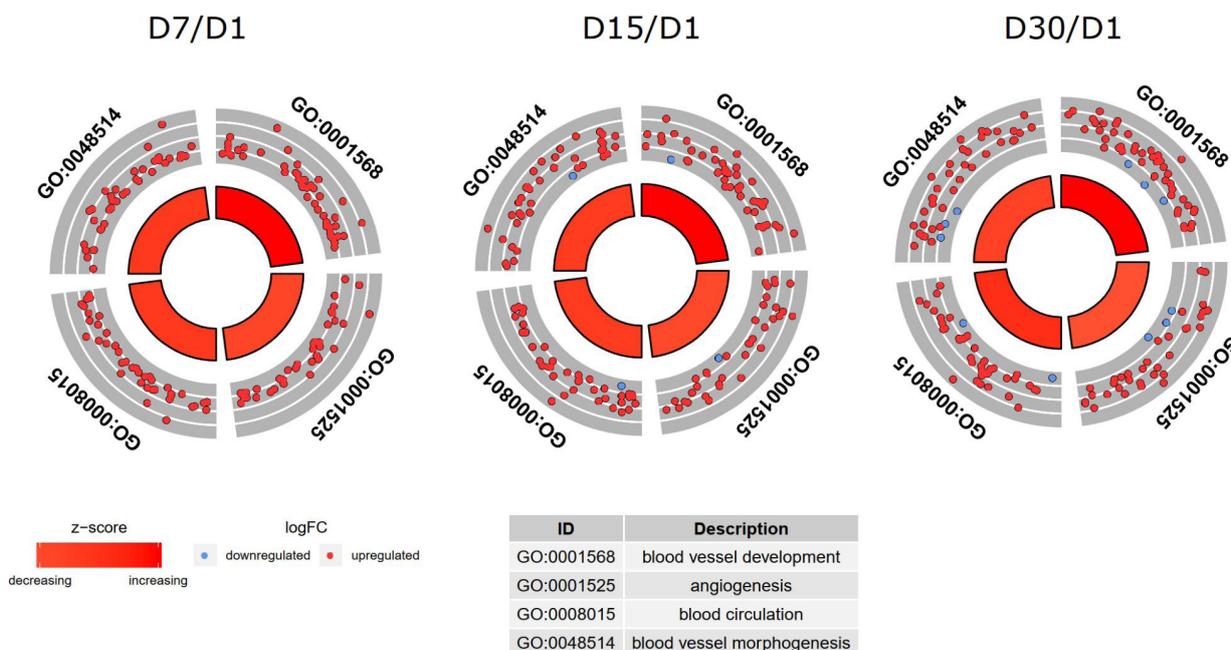


FIGURE 2 The circle plot showing the differently expressed genes and z-score “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms. The outer circle shows a scatter plot for each term of the fold change of the assigned genes. Red circles display 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

sion, Entrez gene IDs and corrected p values of that genes were shown in **table 1**.

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

In Gene Ontology database, genes that formed one particular GO group can also belong to other different GO term categories. By this reason we have explored 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 interaction network was generated among differentially expressed genes belonging to each of selected GO BP terms. Using such prediction method provided us with molecular interaction network formed between protein products of studied genes (**Fig. 5**).

Discussion

Oviductal functions are well established in reproductive process, through facilitating successful embryo growth and conception [4]. The oviduct of the domestic mammals plays a crucial role in providing optimal microenvironment for final gamete maturation and transport, fertilization, and early embryo development, all serving to deliver a competent and healthy conceptus to the endometrium [5]. Oviduct activity is orchestrated by various factors, depending on cyclic dynamics, which crucially affect the success of reproductive function. Moreover, these main functions of oviduct are highly based on its contractibility and the capacity for secreting ovi-

ductal fluid into the lumen. Other oviductal components include hormones, growth factors and their receptors [6,7].

Epithelial oviductal cell lines may be a valuable tool for better understanding and elucidating many biochemical pathways existing in oviducts. Our previous studies have shown various aspects of OECs’ morphological modifications and transcriptomic profile changes during *in vitro* cultivation [8,9]. Therefore, employing a microarray approach in our study, we aimed to investigate the transcriptomic profile of OECs during long-term *in vitro* primary cell culture. From all genes shown in this manuscript, belonging to the five GOs related to the formation of new vessels, six of them (VEGFA, SLIT2, EDNRA, EPAS1, DDAH1 and NPR1) were a common element for all groups and showed the same direction of expression changes during cultivation.

Vascular endothelial growth factor (VEGF) is the main regulator of angiogenesis, inducing endothelial cell proliferation, migration, vascular permeability, and vessel lumen formation [10]. In pigs, 7 isoforms of VEGFA have been confirmed, with predominant role of VEGF120 and VEGF164 isoforms in the female reproductive tissues [11]. The VEGF system includes the VEGF isoforms (VEGFA) and both the Flt-1 (VEGFR-1) and Flk-1/KDR (VEGFR-2) tyrosine kinases, which are high-affinity VEGF receptors [10]. The female reproductive system undergoes many angiogenic changes during each cycle. Previous studies have confirmed that the vascular endothelial growth factor (VEGF) system is crucial

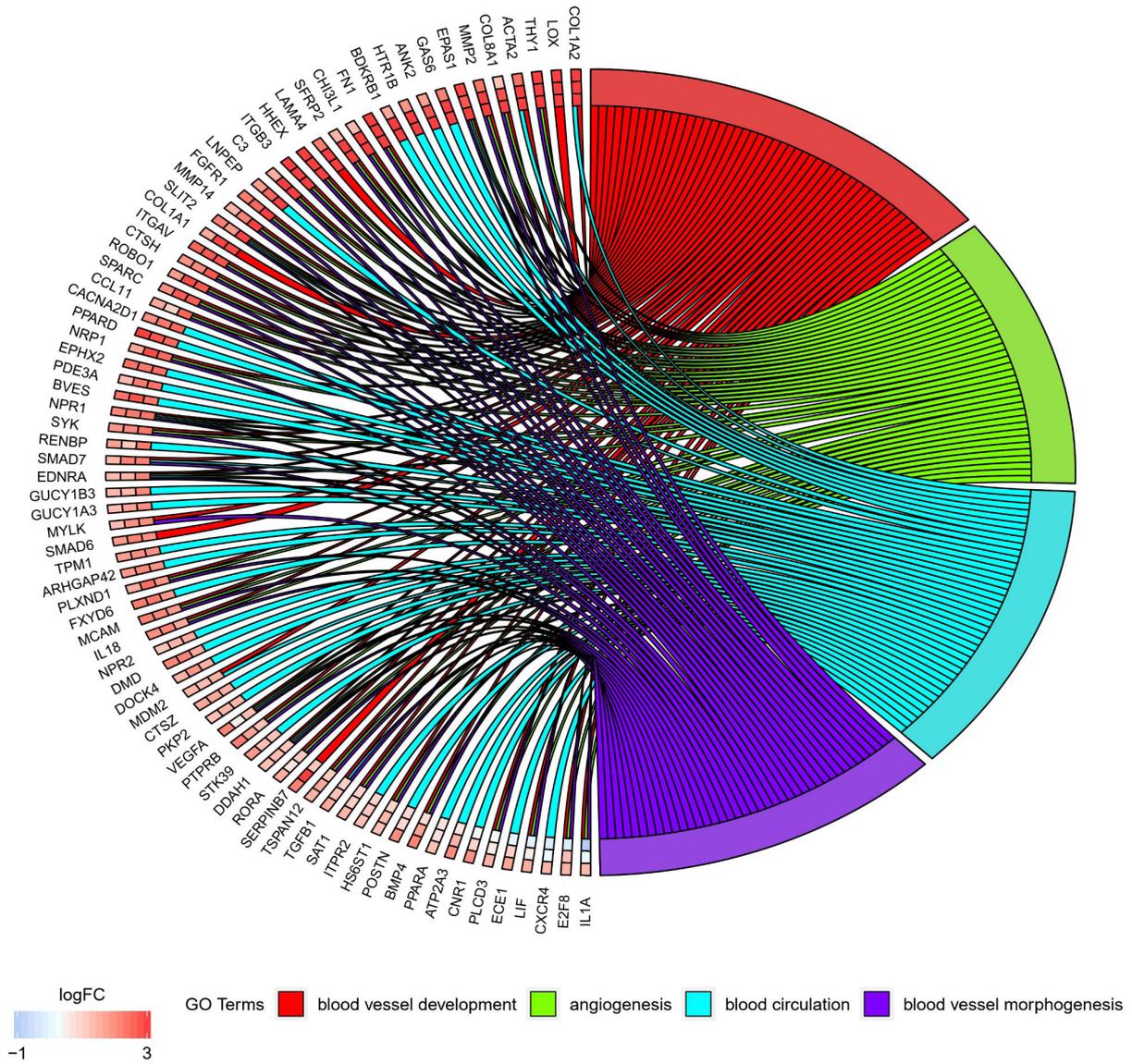


FIGURE 3 The representation of the mutual relationship 10 most upregulated and 10 most downregulated genes that belongs to the “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms. The ribbons indicate which gene belongs to which categories. The middle circle represents logarithm from fold change (LogFC) between D7/D1, D15/D1 and D30/D1 respectively. The genes were sorted by logFC from most to least changed gene

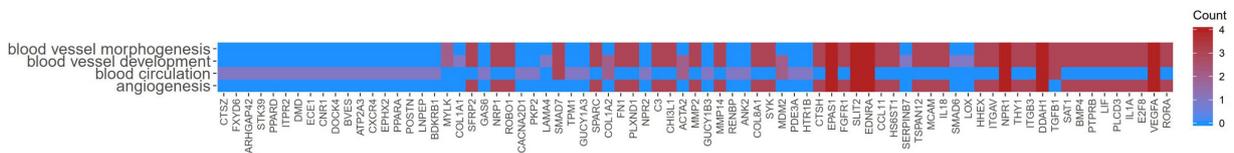


FIGURE 4 Heatmap showing the gene occurrence between differently expressed genes that belongs to “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms. The red color is associated with gene occurrence in the GO Term. The intensity of the color is corresponding to amount of GO BP terms that each gene belongs to

for the regulation of ovarian [12], endometrial [13], and placental [14] angiogenesis. Furthermore, our previous work also showed significant changes in

VEGFA mRNA expression levels in the oocyte-cumulus complexes (COCs) during cell culture [15]. These examples indicate that VEGF system may be postu-

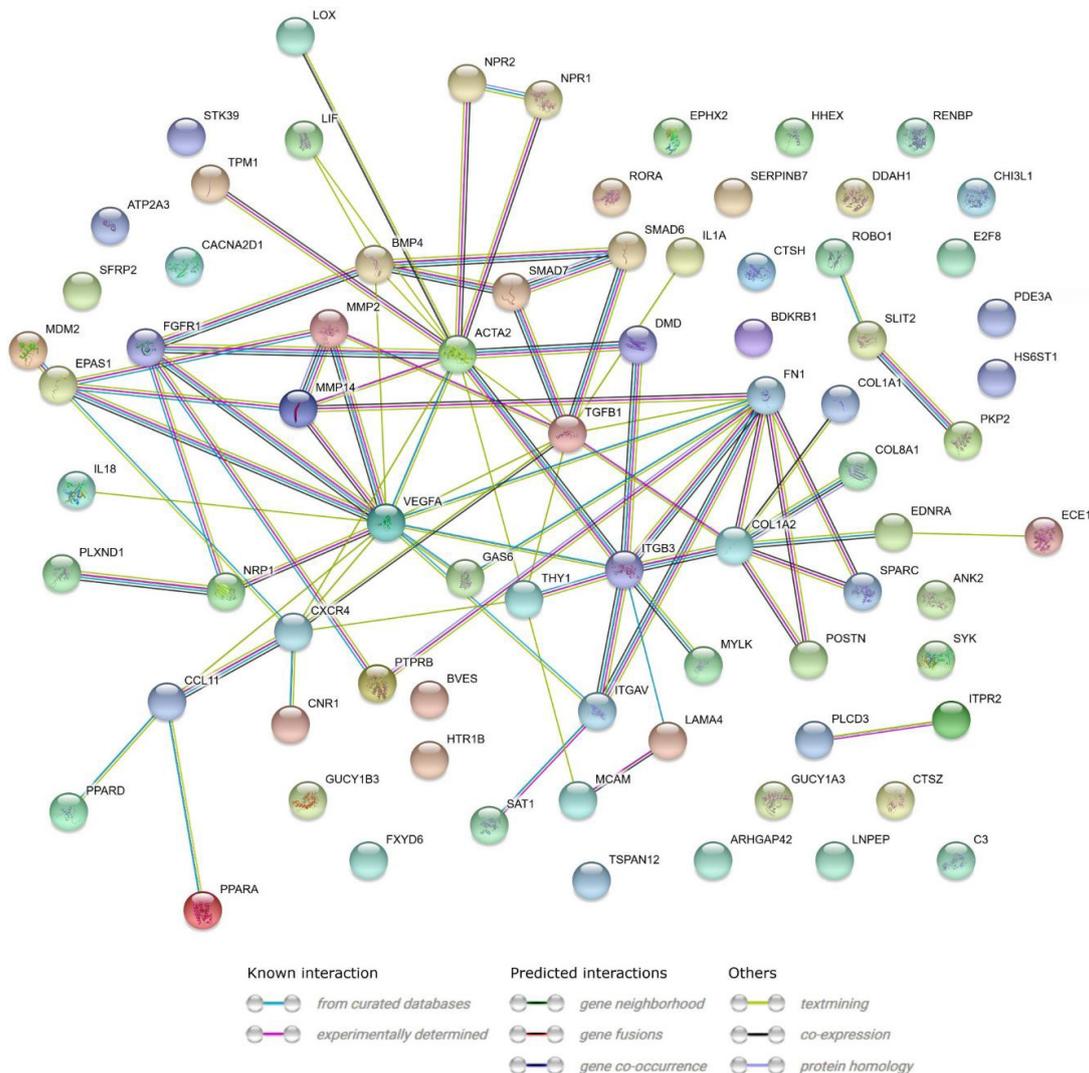


FIGURE 5 STRING-generated interaction occurrence between differently expressed genes that belongs to “angiogenesis”, “blood circulation”, “blood vessel development” and “blood vessel morphogenesis” GO BP terms. The intensity of the edges reflects the strength of interaction score

lated as a key factor involved in the local regulation of potential changes in the microvasculature and a mediator of microvascular permeability in the female reproductive organs of mammals. Lam laboratory characterized the expression dynamics of VEGFA, Flt-1 and KDR in women’s oviducts throughout the estrous and menstrual cycles [16]. These studies confirmed different roles of two VEGF receptors in the oviduct (pivotal role of KDR in oviduct angiogenesis, whereas flt-1 appears to be important in the temporal regulation of oviductal secretion) [16]. Małysz-Cymborska et al. described the influence of insemination and treatment with human chorionic gonadotropin (hCG) and equine chorionic gonadotropin (eCG) on expression of the VEGF system in porcine oviducts [17]. These study showed that insemination alone as well as ovarian stimulation affected the mRNA and protein profiles of the VEGF system in the porcine oviduct. Other investigators, also employing a pig model, demonstrated more

comprehensive analysis by characterizing the gene expression, protein levels and localization of the components of VEGF system in the oviduct throughout the porcine estrous cycle [18]. The investigators described in detail a differential VEGFA, Flt-1 and KDR expression profile observed depending on the oviduct portion, the stage of the estrous cycle and the histological layer. Other research, using equine oviduct explant culture model, have not observed differences in transcript expression levels of VEGFA between Day 0 and Day 6 of culture [19]. Our research, as mentioned above, indicates a higher level of expression during the cell culture.

Efficient process of vascular morphogenesis requires numerous ligands and transcription factors that support the activity of vascular endothelial growth factor’s family. Our studies indicated similar pattern of mRNA expression for three genes that have just such an auxiliary function to VEGFs. Neuropilin 1 (NRP1) plays an important role in

signaling and vascular morphogenesis. Lanahan et al. found that mice with a knock-in mutation that ablates the NRP1 cytoplasmic tail (Nrp1^{cyto}) exhibit normal angiogenesis but impaired developmental and adult arteriogenesis [20]. The effect of VEGFA-induced angiogenesis is enhanced with increased expression of NRP1 co-receptor [20]. Other studies indicated that both placental NRP1 and VEGF were consequently expressed at lower levels in women with pre-eclampsia [21]. Pre-eclampsia (PE) is a clinically important complication during pregnancy, strongly associated with abnormal placentation, endothelial dysfunction and imbalance between pro-angiogenesis and anti-angiogenesis [22]. Dimethylarginine dimethylaminohydrolase type 1 (DDAH1) polymorphisms can also play a significant role in PE susceptibility [23]. DDAH enzyme family plays a role in the regulation of nitric oxide synthesis and release [24]. EPAS1 (Endothelial PAS Domain Protein 1) is the third of mentioned genes, involved in regulation of VEGF expression, seemingly implicated in the development of blood vessels [25].

Mammals have at least three SLIT homologs genes (SLIT1, SLIT2, and SLIT3), which control retinal axon guidance based on their patterns of expression within the developing visual system [26]. Slits signal through Roundabout (ROBO) receptors to play a role in axon guidance and attraction/repulsion of cell migration. In tumorigenesis, SLIT2 attracts vascular endothelial cells and promotes tumor-induced angiogenesis in multiple cancers including those of the reproductive system [27,28]. Additionally, SLIT2 mediates directional migration of malignant cells [29]. SLIT2/ROBO1 signaling plays an important role in placental angiogenesis during normal pregnancy. Hence, investigators analyzed the expression and localization of the SLITs and ROBOs in the oviducts, endometrium [30] and in human tubal placenta [31]. The SLIT2/ROBO1 signaling pathway was investigated as potential factor which may predispose to ectopic tubal pregnancy [30,31]. SLIT2 overexpression promoted vascular remodeling by increasing the diameter of the maternal blood sinusoids and fetal capillaries [31].

In our studies, endothelin receptor type A (EDNRA) also showed increased expression in cells during long-term cultivation, and was included in all discussed GO BP terms associated with the processes of blood vessel formation. The endothelin family contains three 21 amino acid peptides (EDN1, EDN2, and EDN3), which bind to two endothelin receptors, A and B (EDNRA and EDNRB) [32]. EDN1 and EDN2 bind to both EDNRA and EDNRB, whereas EDN3 only binds to EDNRB [33]. The endothelin family was originally found as a group of potent vasoactive peptides, regulating vascular tone and blood pressure [33]. Nevertheless, endothelin participates in various biological processes in the re-

productive system including luteolysis, contraction of myometrium and rupture of the follicle in several mammalian species [33,34]. Other studies indicated that endothelins play a pivotal role in creating optimal oviductal microenvironment, by affecting smooth muscle motility and epithelial NO synthesis [35]. General application defining the roles of these proteins in the functioning of the fallopian tubes has been made by Jeoung et al. and suggest that endothelins are required by the oviduct to facilitate fertilization and early embryonic development [36].

Corresponding author

Bartosz Kempisty PhD, Department of Histology and Embryology, Department of Anatomy, Poznań University of Medical Sciences, 6 Święcickiego St., 60-781 Poznań, Poland Tel./Fax: +48 61 8546418 / +48 61 8546440, e-mail: bkempisty@ump.edu.pl.

Conflict of interest statement

The authors declare they have no conflict of interest.

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