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The blood vessels development, morphogenesis and blood circulation are three ontologic groups highly up-regulated in porcine oocytes before in vitro maturation

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Abstract

The mammalian oocytes undergo significant biochemical and structural modifications during maturation both in vitro and in vivo. These changes involve chromatin reorganization and modification within metabolic status of cytoplasmic organelles. After oocytes' successful maturation the substantially increased storage of RNA was observed. Moreover, the early embryo interaction with maternal endometrial tissue after fertilization is up to now considered as the main marker of proper embryo implantation and early growth. In this study, we first investigated the expression profile of genes involved in blood vessel formation and blood circulation in porcine oocytes before and after in vitro maturation.

The cumulus-oocyte complexes were collected from pubertal Landrace gilts and classified as before in vitro maturation (in Vivo) or after in vitro maturation (in Vitro). The RNA was isolated from these two experimental groups and analyzed using Affymetrix microarrays.

We found an increased expression of genes involved in ontological groups such as "blood circulation" (TPM1, ECE1, ACTA2, EPHX2, EDNRA, NPR2, MYOF, TACR3, VEGFA, GUCY1B3), "blood vessel development" (ANGPTL4, CYR61, SEMA5A, ID1, RHOB, RTN4, IHH, ANGPT2, EDNRA, TGFBR3, MYO1E, MMP14), and "blood vessels morphogenesis" (ANGPT2, as well as other common transcripts) in in Vivo group as compared to decreased expression of these genes in in Vitro group of oocytes.

It has been suggested that investigated genes undergo significant expression before in vitro maturation, when enhanced storage of large amount of RNA takes place. Creating templates for synthesis of proteins is required for formation of fully mature gametes and early embryo growth. Therefore we hypothesized that the processes of vascularization and/or angiogenesis reach a high activity in immature oocytes and are distinct from achievement of maturational stage by oocytes in pigs.

Running title: Regulation of oocytes vascularization in vitro

Keywords: pig, oocytes, ontologic groups, in vitro vascularization, IVM

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Introduction

The mammalian oocytes are surrounded by at least two layers of somatic cells, also known as cumulus cells (CCs). These cells form the architecture of cumulus-oophorus, which displays a substantial role during proper growth of oocytes during folliculogenesis and oogenesis. It was recently well recognized that the oocyte-cumulus oophorus complexes (COCs) play a structural and functional role during female gametes development. Between CCs and oocytes exist specific protein connections, called as gap junctions (GJCs) that are formed by connexins and are crucial for proper transit of small substances between these two cells populations. This unique bi-directional "dialog" seems to be substantial for both oocytes growth and development as well as CCs proliferation and differentiation during antral follicle formation.

Moreover, it was found that COCs undergo significant biochemical and morphological modification during their growth, which was recognized and called as oocyte maturation. The COCs maturation is divided into two stages, described as nuclear and cytoplasmic. Both of them are accompanied by significant cellular changes, which involved chromatin reorganization during reaching of MII stage as well as changes in CCs structure from compact to expanded after maturation.

The molecular changes, determined as the expression profile of clusters for genes during folliculogenesis and oogenesis within single oocyte as well as biochemical pathways activated during oocytes maturation are well defined. However, the marker genes and/or clusters of genes that regulate the morphological modification of COCs are still not entirely known. The regulation of cellular morphogenesis may "open new gates" in description of early embryogenesis, especially preimplantation embryo development. Furthermore, the structural modifications of maternal endometrial tissue and embryo receptivity during implantation process are still poorly recognized.

The ability of embryo to formation of blood vessels networks may be a main mechanism during successful implantation, decidualization, and early embryo – maternal interaction. Therefore, this study was aimed to investigate the expression profile of "blood vessels morphogenesis", "development" and "blood circulation" ontology groups during porcine COCs *in vitro* maturation (IVM).

Material and methods

Animals

A total of 45 pubertal crossbred Landrace gilts, bred on a local, commercial farm, were used in this study. They had a mean age of 155 days (range 140 – 170 days) and a mean weight of 100 kg (95-120 kg). All of the animals were housed under identical conditions and fed the same forage (depending on age and reproductive status). The experiments were approved by the Local Ethic Committee.

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

Ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory at 38° C in 0.9% NaCl within 40 min. To provide optimal conditions for subsequent oocyte maturation and fertilization *in vitro*, the ovaries of each animal were placed in 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. Single large follicles (>5mm) were then opened by puncturing with a 5 ml syringe and 20-G needle in a sterile Petri dish, and COCs were recovered. The COCs were washed three times in modified PBS supplemented with 36 μg/ml pyruvate, 50 μg/ml gentamycine, and 0.5 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). COCs were selected under an inverted microscope Zeiss, Axiovert 35 (Lübeck, Germany), counted, and morphologically evaluated. Only COCs of grade I with homogeneous ooplasm and uniform, compact cumulus cells were considered for the following steps of the experiment, resulting 300 grade I oocytes (3 x n=50 in Vivo group, 3 x n=50 in Vitro group).

Assessment of oocyte developmental competence by BCB test

To perform the BCB staining test, oocytes were washed twice in modified Dulbecco PBS (DPBS) (Sigma-Aldrich, St. Louis, MO) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), 0.4% BSA [w/v], 0.34 mM pyruvate, and 5.5 mM glucose (DPBSm). Thereafter, they were treated with 13 μM BCB (Sigma-Aldrich, St. Louis, MO) diluted in DPBSm at 38.5° C and 5% CO_2 for 90 min. After treatment, the oocytes were transferred to DPBSm and washed twice. During the washing procedure, the oocytes were examined under an inverted microscope and classified as either stained blue (BCB^+) or remained colorless (BCB^-). Immature oocytes have compact cumulus cell layers that require removal for further oocyte evaluation. Next, the BCB^+ COCs were first incubated with bovine testicular hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA) for 2 min at 38° C to separate cumulus and granulosa cells. Cells were then removed by vortexing the BCB^+ COCs in 1% sodium citrate buffer followed by mechanical displacement using a small-diameter glass micropipette. Only the granulosa cell-free BCB^+ oocytes were used for subsequent microarray analysis (in Vivo) or IVM followed by microarray analysis (in Vitro).

In vitro maturation of porcine COCs

After the first BCB test, the BCB^+ oocytes were cultured in Nunclon™Δ 4-well dishes in 500 μl of standard porcine IVM culture medium TCM-199 (tissue

culture medium) with Earle's salts and *L*-glutamine, (Gibco BRL Life Technologies, Grand Island, NY, USA) supplemented with 2.2 mg/ml sodium bicarbonate (Nacalai Tesque, Inc., Kyoto, Japan), 0.1 mg/ml sodium pyruvate (Sigma-Aldrich, St. Louis, MO, USA), 10 mg/ml BSA (bovine serum albumin), (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/ml cysteine (Sigma-Aldrich, St. Louis, MO, USA), 10% filtered porcine follicular fluid (v/v), and gonadotropin supplements at final concentrations of 2.5 IU/ml hCG (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/ml eCG (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38° C under 5% CO_2 in air for 22h, and then for additional 22h in medium without hormones. After cultivation, the BCB staining test was performed again, and BCB^+ oocytes were used for further experiments.

RNA extraction from porcine oocytes

Oocytes investigated before and after IVM were pooled into three independent samples for each experimental group. 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 were taken.

Microarray expression analysis

Total RNA (100 ng) from each pooled sample was subjected to two rounds, 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). Then, microarrays were 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. The 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 to the selection of genes involved in morphogenesis and cellular differentiation. The differentially expressed gene list (separated for up- and down-regulated genes) was uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery), where 21 genes belonging to "blood circulation" (GO:0008015), "blood vessel development" (GO:0001568) and "blood vessel morphogenesis" (GO:0048514) GO terms were obtained.

Expression data of these genes were subjected to a hierarchical clusterization procedure, and their expression values were presented as a heatmap. Besides predicting interactions, DAVID software also allowed performance of functional enrichments of GO terms based on previously uploaded gene sets from the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" GO BP terms.

Interactions between differentially expressed genes/proteins belonging to "blood circulation", "blood vessel development" and "blood vessel morphogenesis" ontology groups were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes). The list of gene names was used as 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 analysis 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 after IVM (in Vitro) in relation to transcriptome profile of freshly isolated oocyte (in Vivo), we performed whole gene expression analysis by Affymetrix® Porcine Gene 1.1 ST Array. In such assay expression of more than 12258 porcine transcripts was examined. The genes for which the fold change was higher than the cut-off value (fold>|2|) and corrected p value (adj $p<0.05$), were considered as differentially expressed. From the whole transcript that consists of 419 different genes, 379 genes were down-regulated and 40 genes were up-regulated in relation to the oocyte transcriptome before IVM.

Among these genes, 21 belonging to "blood circulation", "blood vessel development" and "blood vessel morphogenesis" gene ontology biological process terms were extracted by DAVID (Database for Annotation, Visualization and Integrated Discovery) software. Up- and down-regulated gene sets were subjected to DAVID searching separately, and only those, where adj. p values were lower than 0.05 were selected (Table1). These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmap (Fig. 1).

Genes from one particular GO group will belong also to other different GO term categories. The genes that are shared by the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" GO BP terms were presented as table (Table 2) and as concept-gene network generated with GeneAnswers R package (Fig. 2).

TABLE 1 Fold changes, adjusted p values and ENTREZ gene ID of differentially expressed genes belonging to the "blood circulation", "blood vessel development" and "blood vessel morphogenesis". Symbols and names of the selected genes are also shown

Official gene symbol	Fold change	adj.p. value	ENTREZ GENE ID
ACTA2	0.26255565	1.596737e-03	59
ANGPT2	0.23738329	1.061779e-03	285
ANGPTL4	0.18363131	5.134222e-04	51129
CYR61	0.08065704	7.535476e-05	3491
ECE1	0.39538773	1.177804e-03	1889
EDNRA	0.16693903	1.854220e-03	1909
EPHX2	0.15013469	1.340845e-04	2053
GUCY1B3	0.40796768	1.855975e-02	2983
ID1	0.33547314	3.974331e-03	3397
IHH	0.30499584	5.512614e-04	3549
MMP14	0.48872115	3.806042e-02	4323
MYO1E	0.32688656	1.132330e-03	4643
MYOF	0.45804453	2.305619e-03	26509
NPR2	0.30252785	1.132330e-03	4882
RHOB	0.33791290	2.898885e-02	388
RTN4	0.23137773	2.749581e-02	57142
SEMA5A	0.35339172	1.092396e-03	9037
TACR3	0.11506032	1.480362e-04	6870
TGFBR3	0.19652224	4.059785e-04	7049
TPM1	0.43396311	1.632742e-03	7168
VEGFA	0.06968939	1.912689e-03	7422

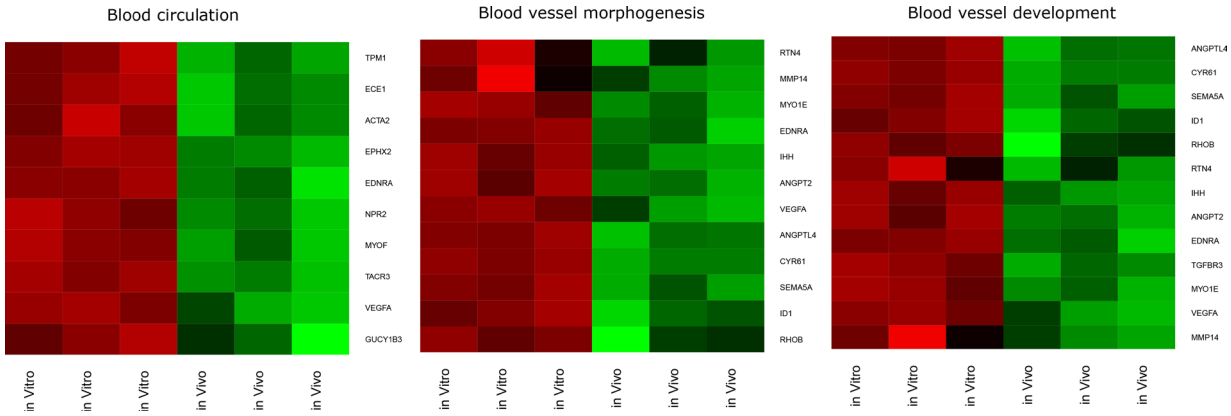

FIGURE 1 Heatmap representation of differentially expressed genes belonging to the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" categories from GO.BP database. Arbitrary signal intensity acquired from microarray analysis is represented by colours (green, higher; red, 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)

TABLE 2 The list of differently expressed genes from the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" ontology groups

	all	blood circulation	blood vessel development	blood vessel moprhogenesis
1	ACTA2	ACTA2		
2	ANGPT2		ANGPT2	ANGPT2
3	ANGPTL4		ANGPTL4	ANGPTL4
4	CYR61		CYR61	CYR61
5	ECE1	ECE1		
6	EDNRA	EDNRA	EDNRA	EDNRA
7	EPHX2	EPHX2		
8	GUCY1B3	GUCY1B3		
9	ID1		ID1	ID1
10	IHH		IHH	IHH
11	MMP14		MMP14	MMP14
12	MYO1E		MYO1E	MYO1E
13	MYOF	MYOF		
14	NPR2	NPR2		
15	RHOB		RHOB	RHOB
16	RTN4		RTN4	RTN4
17	SEMA5A		SEMA5A	SEMA5A
18	TACR3	TACR3		
19	TGFBR3		TGFBR3	
20	TPM1	TPM1		
21	VEGFA	VEGFA	VEGFA	VEGFA

STRING-generated interaction network was generated among differentially expressed genes belonging to the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" GO BP terms. Applied prediction methods used text mining, co-expression, and experimentally observed interactions (Fig. 3).

Discussion

The proper COCs maturation is necessary for formation of fully fertilizable oocyte, formation of zygotes as well as proper embryos growth in preimplantation stage. It is well recognized that oocytes' maturation stage is divided into two phases, such as nuclear and cytoplasmic. Moreover it was defined that proper regulation of these processes is required for complete maturation of oocytes. Our previous studies have shown various aspects of changes occurring in oocytes during maturation [1-3] and impact of environmental changes on these processes [4-5]. Morphological and biochemical alterations associated with the ability of embryo to formation of blood vessels networks seem to play pivotal role in successful implantation, decidualization and early embryo – maternal interaction.

Therefore, employing a microarray approach in our study, we aimed to investigate the transcriptome profile of COCs before (in Vivo) and after (in Vitro) IVM. From all analyzed genes which showed different expression patterns, 21 genes belong to "blood circulation", "blood vessel development" and "blood vessel morphogenesis" gene ontology biological process terms. Obtained results indicate decreased mRNA expression of all analyzed genes associated with formation of blood vessels networks after IVM comparing to transcript levels of freshly isolated oocyte. From all of 21 presented, down-regulated genes, two of them: *VEGFA* and *EDNRA*, exhibit inhibitory effect to gene expression in all three investigated GO BP terms. Vascular endothelial growth factor A (VEGFA) is well established as a critical regulator of angiogenesis in the ovulatory follicle [6]. Moreover, in addition to mediation of endothelial cells angiogenic process, studies with human ovarian microvascular endothelial cells (hOMECs) proved that VEGFA promote endothelial cell migration [7]. Li et al. (2016) indicate improved quality and survival rate of subcutaneously-transplanted mouse ovarian tissue in VEGF coupled with FGF2-treated subjects [8]. Furthermore, IVM of bovine oocytes supplemented with VEGF results in an improvement of cytoplasmic maturation, with a positive impact on oocyte developmental capacity [9]. Our results indicate the lowest fold change of *VEGFA* transcript levels from all differentially ex-

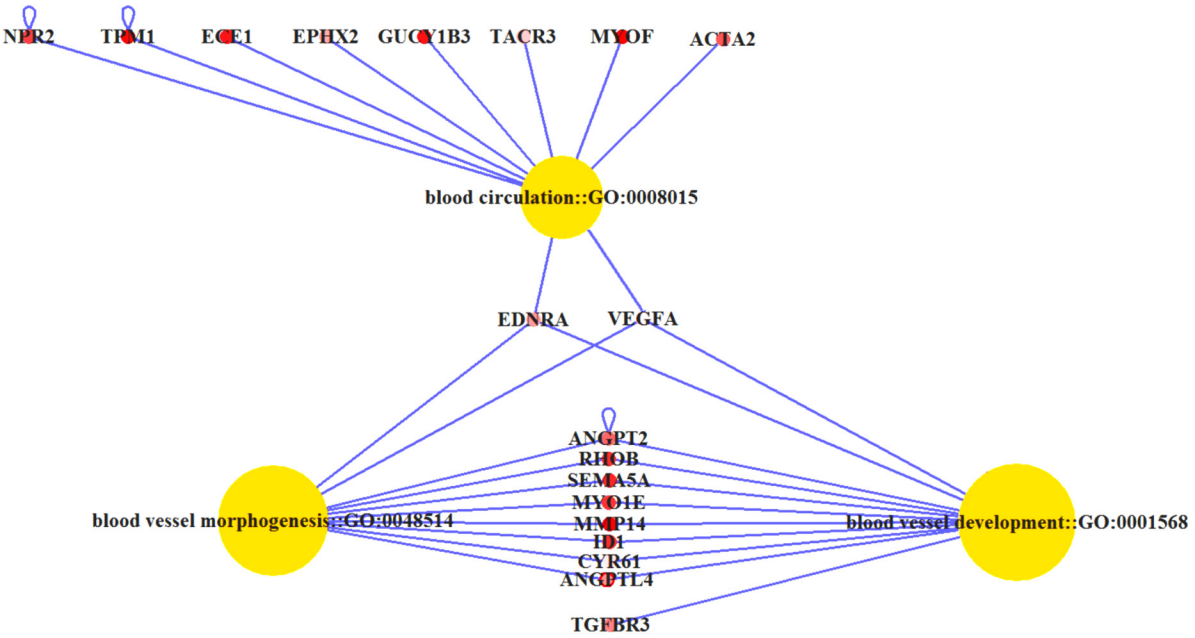

FIGURE 2 Concept-gene network which shows which differently expressed genes from "blood circulation", "blood vessel development" and "blood vessel morphogenesis" that are shared between those categories. The genes belonging to the same GO terms are sorted next to each other. The intensity of the red color of the dots represents the fold change

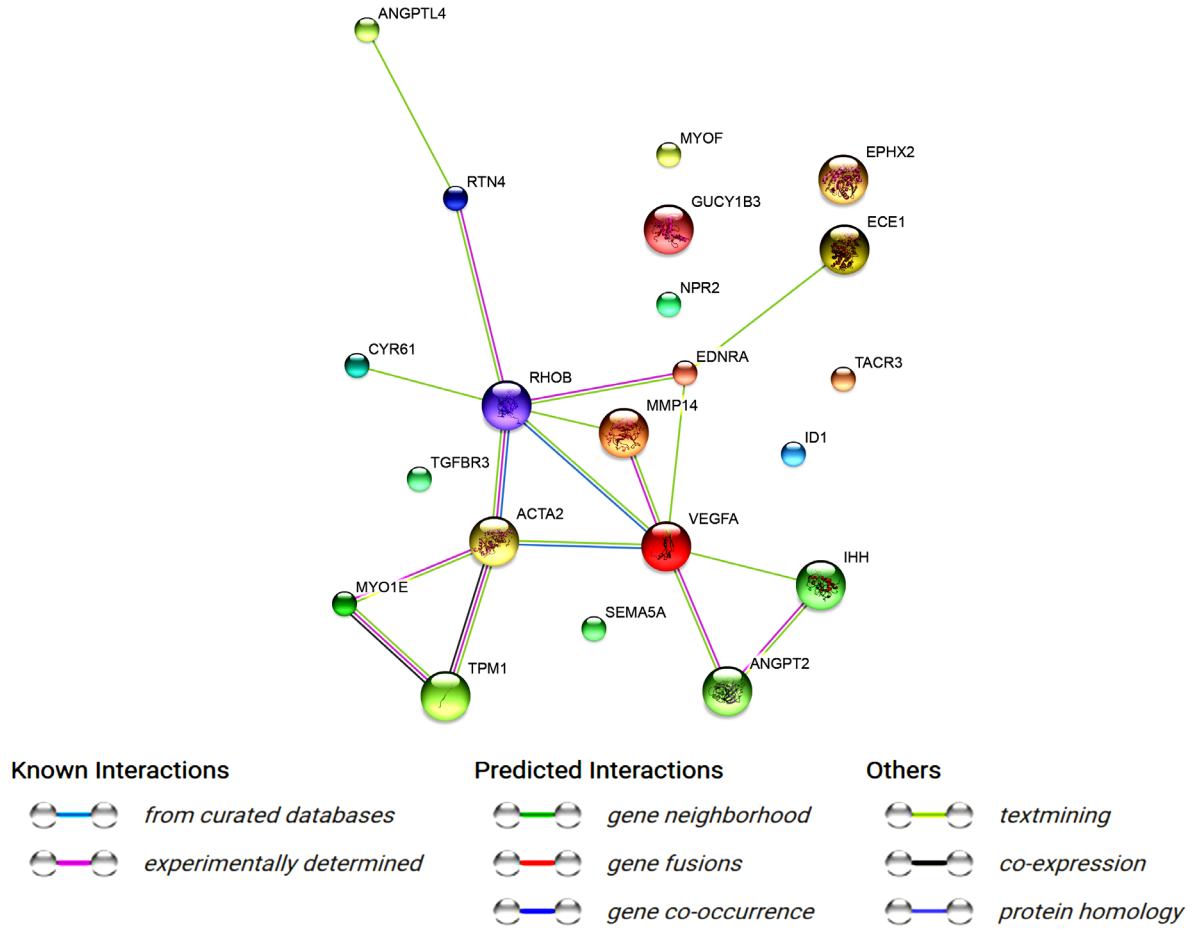

FIGURE 3 STRING-generated interaction network among differentially expressed genes belonging to the "blood circulation", "blood vessel development" and "blood vessel morphogenesis" ontology groups. Applied prediction methods: text mining, co-expression, experimentally observed interactions

pressed genes analyzed in this study. Endothelin receptor A (EDNRA) were also down-regulated after IVM in all three investigated GO BP terms. The endothelins (ETs) have a wide variety of biological effects, not only in the cardiovascular system but also in the nonvascular system, in various tissues and cell types, which are mediated by two classes of G-protein coupled receptors: endothelin receptor A and B [10]. Data from Ko et al. (2006) studies suggest that endothelin-2 (EDN-2), expressed in the granulosa cells immediately before ovulation, induces follicular rupture by constricting periovulatory follicles [11]. Induced by EDN-2 follicular smooth muscle contraction, being the driving force of the follicular rupture, is facilitated via EDNRA [12]. These findings, taken together with our results, highlight the important role of endothelins and their receptors in the ovulatory process.

Furthermore heatmaps, showing obtained in this study results, indicate the variable mRNA expression profile of 10 genes belonging to both "blood vessel development" and "blood vessel morphogenesis" GO terms. These investigated ontology groups commonly include genes such as: angiopoietin-2 (*ANGPT2*), angiopoietin-like 4 (*ANGPTL4*), cysteine-rich angiogenic inducer 61 (*CYR61*), inhibitor of DNA binding 1 (*ID1*), indian hedgehog (*IHH*), matrix metalloproteinase 14 (*MMP14*), myosin 1E (*MYO1E*), RAS homolog gene family, member 14 (*RHOB*), reticulon 4 (*RTN4*) and semaphorin 5A (*SEMA5A*).

The angiopoietin family coupled with tyrosine kinase receptors (Tie1 and Tie2) create angiopoietin/Tie system which play pivotal role in blood vessel formation [13]. Holash et al. (1999) concluded that their observations indicate that angiopoietin-1 was antiapoptotic for cultured endothelial cells and expression of its antagonist angiopoietin-2 was induced in the endothelium of co-opted tumor vessels before their regression [14]. ANGPT2 acts in angiogenesis as antagonist for ANGPT1, inhibiting ANGPT1-promoted Tie2 signaling, which is critical for blood vessel maturation and stabilization. ANGPT2 modulates angiogenesis in a cooperative manner with another important angiogenic factor, VEGF [14-15]. Furthermore, Hata et al. (2002) suggested that there is a difference in the angiopoietin/Tie2 gene expression between physiological and pathological angiogenesis in the ovary [16]. Angiopoietin-like proteins (ANGPTLs) are a family of proteins structurally similar to the angiopoietins. In contrast to angiopoietins, all angiopoietin-like proteins (ANGPTL1-8) do not bind to the tyrosine kinase receptors, because they have been generally considered orphan ligands [17-18]. ANGPTL4 protein in mice is most highly expressed in white and brown adipose tissues and to a much lesser extent in other tissues such as ovary, heart, liver, skeletal muscle and intestine [19]. Liu et al. (2016) indicate that aberrant expression patterns of *ANGPTL1* and *ANGPTL2* transcripts in cumulus cells during oocyte maturation is potentially associated with impaired oocyte developmental competence in polycystic ovary syndrome (PCOS) [20].

Our results indicate the highest fold change from all investigated genes for matrix metalloproteinase 14 (*MMP14*) mRNA, which is Zn(2+)-binding endopeptidase that degrades various components of the extracellular matrix [21]. Another important factor involved in the processes of "blood vessel development" and "blood vessel morphogenesis" is cysteine-rich protein 61 (CYR61). This is an important molecule which was shown to participate in a number of key cellular processes, including cell differentiation, adhesion, migration, proliferation, wound healing, and angiogenesis. The *CYR61* gene is key signaling factor involved in angiogenesis that is prerequisite for the initial process of fracture healing [22].

Some of the analyzed transcripts fall into "blood circulation" GO term. Smooth muscle aortic alpha-actin (ACTA2) seems to play an important role in this process. During oocyte maturation actin cytoskeleton participates in the formation of the cortical actin cap, increasing the cytoplasmic actin density [23] and the thickness of cortical actin [24]. The actin cytoskeleton is the main driving force for asymmetric division in mammalian oocytes. Endothelin-converting enzyme-1 (ECE-1) is the enzyme predominantly responsible for producing active endothelin-1 (EDN-1), previously described in this research. ECE-1 exists as four isoforms, ECE-1a to ECE-1d, which are generated from a single gene by the use of alternative promoters [25].

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Competing interests
The authors declare they have no conflict of interest

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