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Epithelium morphogenesis and oviduct development are regulated by significant increase of expression of genes after long-term in vitro primary culture – a microarray assays

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Abstract

The correct oviductal development and morphogenesis of its epithelium are crucial factors influencing female fertility. Oviduct is involved in maintaining an optimal environment for gametes and preimplantation embryo development; secretory oviductal epithelial cells (OECs) synthesize components of oviductal fluid. Oviductal epithelium also participates in sperm binding and its hyperactivation. For better understanding of the genetic bases that underlay porcine oviductal development, OECs were isolated from porcine oviducts and established long-term primary culture. A microarray approach was utilized to determine the differentially expressed genes during specific time periods. Cells were harvested on day 7, 15 and 30 of in vitro primary culture and their RNA was isolated. Gene expression was analyzed and statistical analysis was performed. 48 differentially expressed genes belonging to "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of branching structure" and "morphogenesis of branching epithelium" GO BP terms were selected, of which 10 most upregulated include BMP4, ARG1, SLIT2, FGFR1, DAB2, TNC, EPAS1, HHEX, ITGB3 and LOX. The results help to shed light on the porcine oviductal development and its epithelial morphogenesis, and show that after long-term culture the OECs still proliferate and maintain their tube forming properties.

Running title: Genes of oviductal epithelium morphogenesis in in vitro culture

Keywords: pig, oviducts, epithelial cells, microarray, cells morphogenesis

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Introduction

The correct oviductal morphogenesis and its epithelium development are crucial factors influencing female fertility, as they are involved in both fertilization and early embryo development. However, genetic regulation of these processes in pigs remains to be elucidated.

The oviduct derives from the Müllerian duct, which originates from the intermediate mesoderm and starts to develop around E11.5 in mice [1]. Initially, embryos have both Müllerian and Wolffian ducts, thus they are anatomically undistinguishable. However, after the sexual differentiation, the Wolffian ducts degenerate in female embryos and the Müllerian duct further develops. In male embryos this situation is opposite [2]. Significant genes involved in this process, such as *Lim1*, *Lhx1*, *Pax2*, *Emx2*, *Wnt4*, *Wnt9b*, *Tcf2*, *Dach1* and *Dach2,* have been distinguished [2, 3, 4]. Further development results in oviduct, uterus and upper vagina formation and subsequent epithelial differentiation, which likely remains under control of the *Hox* genes, especially *Hoxa10*, *Hoxa11* and *Hoxa13*, as well as *Wnt7a* gene [2, 3]. The timing of epithelial differentiation varies between species; in humans it occurs in the fetal stage, while in mice it takes place postnatally [5]. Epithelial morphogenesis involves several events, such as apicobasal polarity and lumen formation, which can occur through epithelial folding, wrapping or invagination. Among many signaling pathways engaged in tubulogenesis, the integrin-mediated signaling pathway and FGF signaling pathway seem to be one of the most significant [6].

Four oviductal regions can be distinguished: the infundibulum, the ampulla, the isthmus and the uterotubal junction. Oviduct has two muscle coats (longitudinal and circular), a mucosa layer, which exhibits folds with a branch-like structure, and epithelial lining [7, 8]. There are two types of epithelial cells in the oviduct: ciliated and secretory cells. The ciliated cells play a significant role in transporting gametes and the embryo, and their characteristics may vary between pig breeds [9, 10]. Secretory cells synthesize components of oviductal fluid, such as nutrients, cytokines, immunoglobulins and embryotropic factors, thus participate in providing an optimal environment for fertilization and promote the early embryo development [11]. The presence of stem-like cells in the oviductal epithelium has also been reported [12].

The oviductal role is invaluable in a successful pregnancy, however its detailed mechanism is beyond the scope of this paper. Aside from providing an optimal environment for gametes and preimplantation embryo development, oviduct guides sperm to fertilization site through rheotaxis, thermotaxis and chemotaxis [9]. Oviductal epithelial cells also bind sperm [13] and are involved in its hyperactivation [14].

For better understanding of the genetic bases that underlay porcine oviductal development and morphogenesis of its epithelium, a long-term primary culture of porcine oviductal epithelial cells has been established, with microarray approach utilized to determine the differentially expressed genes during specific time periods. Such studies may be helpful in optimizing assisted reproduction techniques in pigs, which still remain less successful than in other species [15], and in understanding genetic events that lead to epithelial tube formation in pigs.

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 selection by examination of genes involved in oviductal epithelium morphogenesis. 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. 32/2012.

Results

Whole transcriptome profiling by Affymetrix microarray allows analyzing gene expression changes between 7, 15 and 30 days of porcine oviductal epithelial cells culture. By Affymetrix® Porcine Gene 1.1 ST Array Strip we examined expression of 12257 transcripts. Genes with fold change higher then abs (2) and wit corrected p value lower than 0.05 were considered as differentially expressed. This set of genes consists of 2533 different transcripts. The first detailed analysis based on GO BP the identification of differentially expressed genes belonging to the significantly enrichment GO BP terms.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of gene ontology biological process term (GO BP) that contains differently expressed transcripts. Up and down regulated gene sets were subjected to DAVID searching separately and only gene

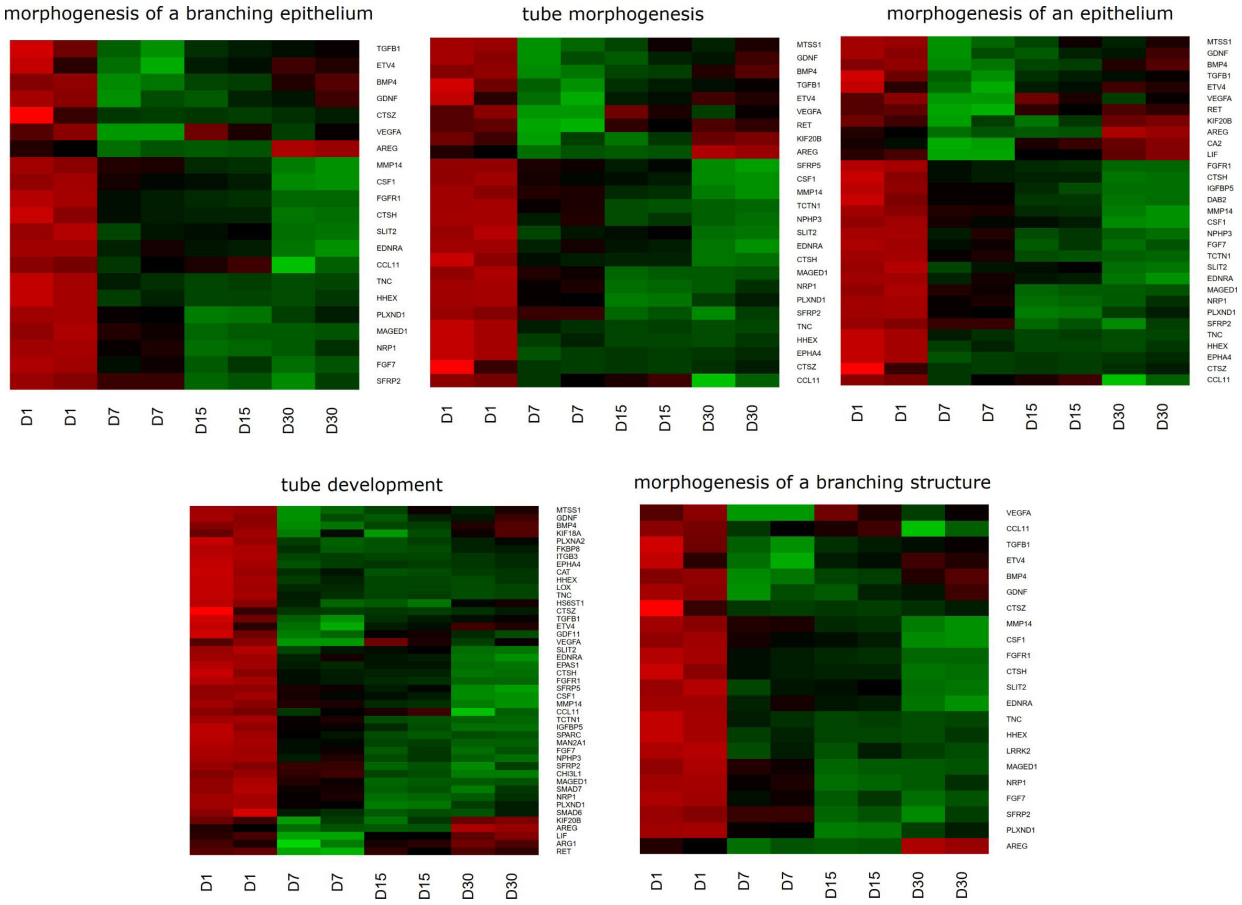
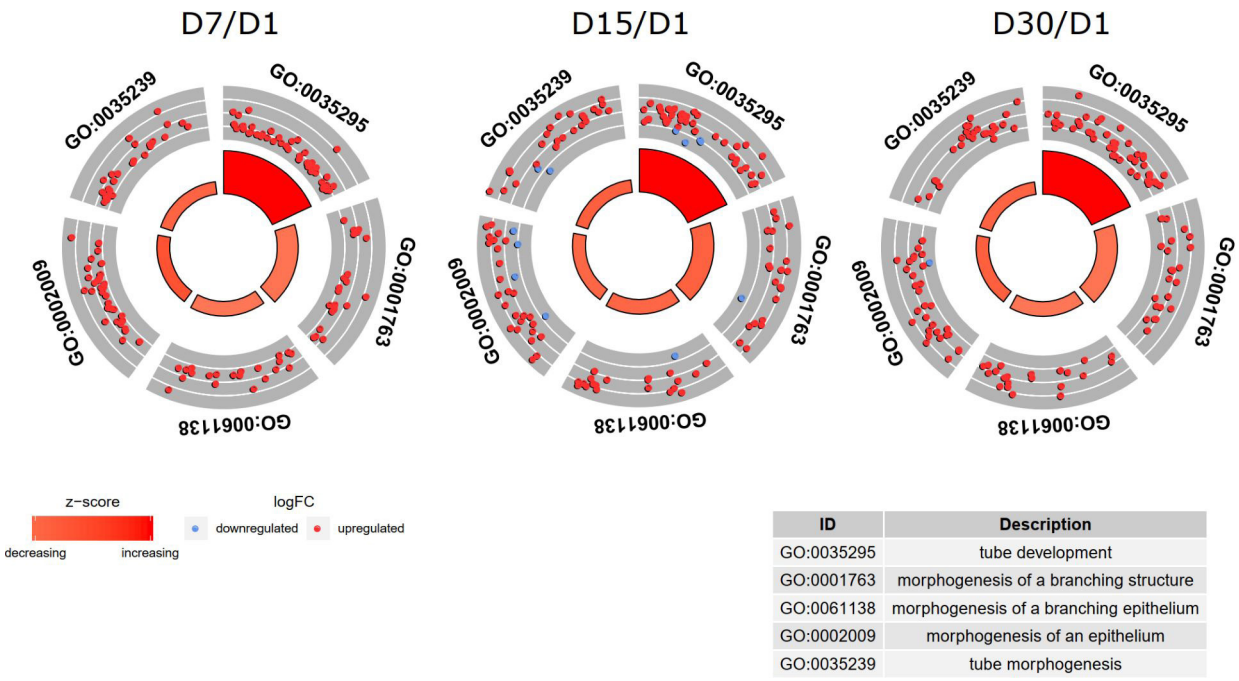


FIGURE 1 Heat map representation of differentially expressed genes belonging to the to "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. Arbitrary signal intensity acquired from 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	Gene ID
BMP4	4,274223	3,01201	1,551199	4,00E-05	9,03E-05	0,007449	100113425
ARG1	4,347305	1,136755	0,748778	0,000869	0,698683	0,310832	100628107
SLIT2	4,503981	3,402733	7,662301	0,000117	0,000211	1,00E-05	100515328
FGFR1	4,599714	5,650775	8,942976	1,33E-05	3,63E-06	6,88E-07	100153248
DAB2	4,822568	8,861786	15,85513	0,000774	0,000116	2,25E-05	100519746
TNC	6,57267	8,352323	8,773467	1,83E-05	4,91E-06	2,42E-06	397460
EPAS1	7,629974	6,655106	16,82792	8,57E-06	5,17E-06	5,84E-07	100037272
HHEX	9,447447	11,82395	11,30739	2,69E-05	9,59E-06	6,25E-06	397232
ITGB3	12,67365	13,15962	10,9347	4,27E-06	1,55E-06	8,03E-07	397063
LOX	29,0517	43,84613	46,65297	7,88E-06	2,86E-06	1,06E-06	100525278

sets where adj. p value were lower than 0.05 were selected. The DAVID software analysis showed that differently expressed genes belongs to 657 Gene ontology terms. In this paper we focused on 48 genes that belongs to "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (**Fig. 1**). The gene symbols, fold changes in expression, Entrez gene IDs and corrected p values



ID	Description
GO:0035295	tube development
GO:0001763	morphogenesis of a branching structure
GO:0061138	morphogenesis of a branching epithelium
GO:0002009	morphogenesis of an epithelium
GO:0035239	tube morphogenesis

FIGURE 2 The circle plot showing the differently expressed genes and z-score of the "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" 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

of that genes were shown in **table 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**).

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

To investigate which genes are involved in porcine oviductal development and its epithelial morphogenesis, we utilized the whole transcriptome profiling method in porcine OEC long-term primary culture, and compared gene expression on day 7, 15 and 30. We have found 48 differentially expressed genes belonging to "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of branching structure" and "morphogenesis of branching epithelium" GO BP terms. However we would like to focus on 10 genes that exhibited the most significant increase in expression. These genes include *BMP4*, *FGFR1*, *TNC*, *ITGB3*, *DAB2*, *SLIT2*, *HHEX*, *EPAS1*, *LOX* and *ARG1*, most of which are involved in multiple signaling pathways associated with many developmental processes in the entire organism.

Amongst the aforementioned genes, the *BMP4* gene can be distinguished. It is a part of TGFβ superfamily of factors and is involved in many developmental processes, such as cartilage and bone formation. It acts via SMAD signaling pathway, influencing gene expression [19]. Also, the role of *BMP4* in reproduction has been reported, as it participates in germ cell proliferation, migration, gametogenesis, folliculogenesis and steroidogenesis [20]. Its expression occurs in the rat theca cells and ovarian and uterine epithelium, in mice granulosa and theca cells and in human oocyte and granulosa cells [21, 22, 23, 24]. Moreover, Tanwar et al. [24] reported *BMP4* expression in murine oviductal epithelial cells, while we observed an increase in this gene's expression in porcine OECs on day 7 and 15 of primary *in vitro* culture. This suggests that this factor may be involved in OEC development as well, aside from its multifunctional role during other tissues morphogenesis.

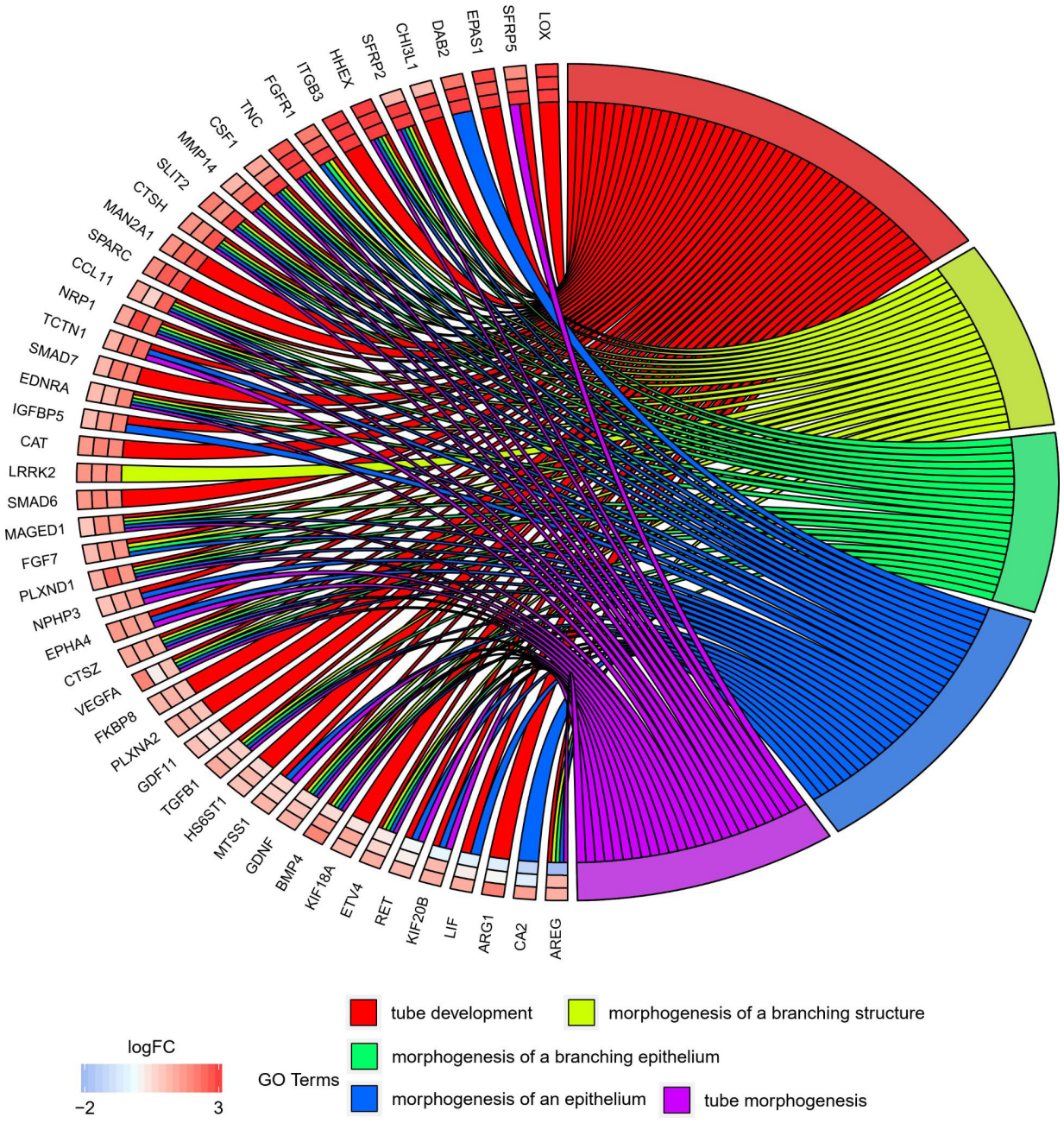


FIGURE 3 The representation of the mutual relationship of differently expressed genes that belongs to the "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. The ribbons indicate 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

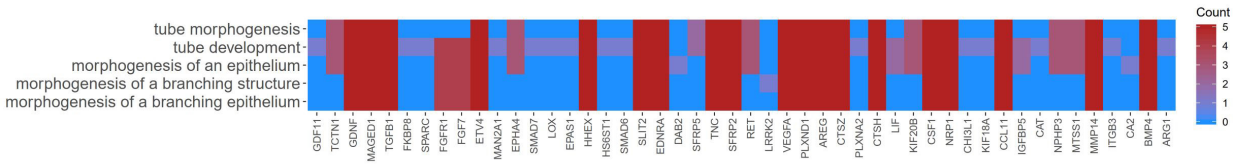


FIGURE 4 Heatmap showing the gene occurrence between differently expressed genes that belongs to the "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. The red color is associated 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

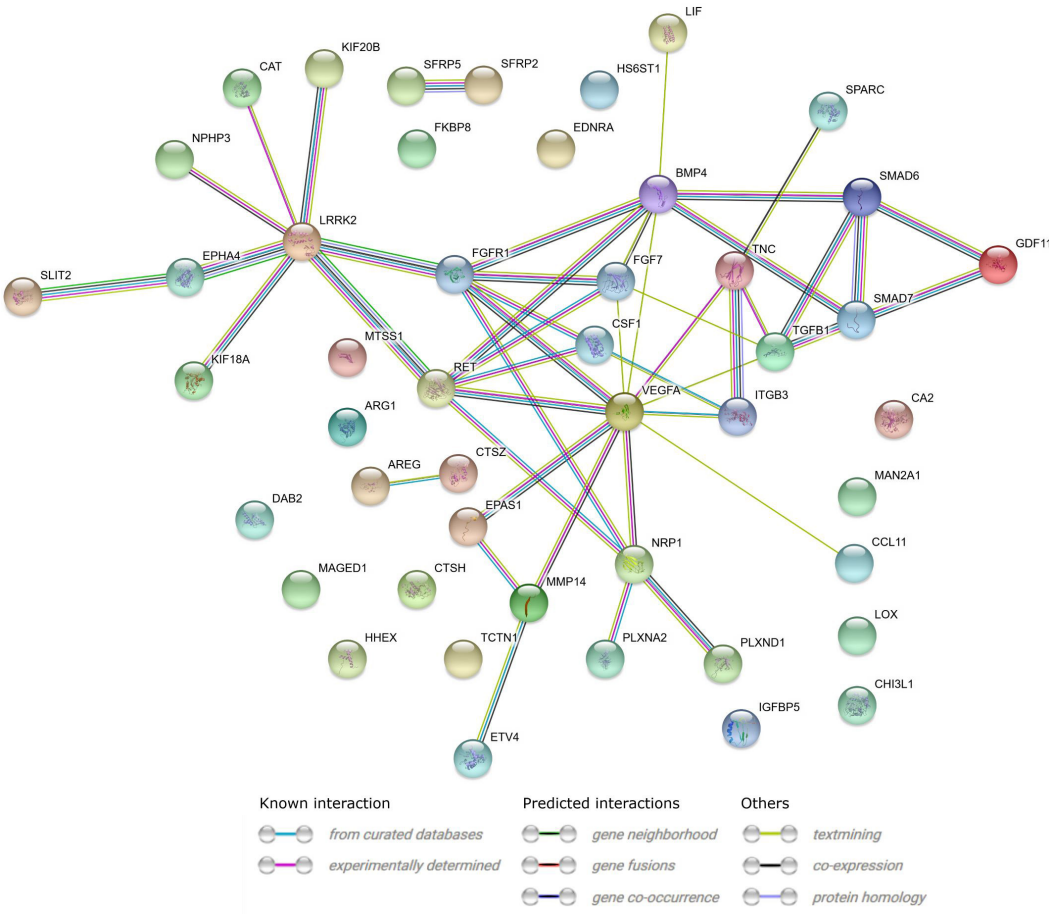


FIGURE 5 STRING-generated interaction occurrence between differently expressed genes that belongs to the "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. The intensity of the edges reflects the strength of interaction score

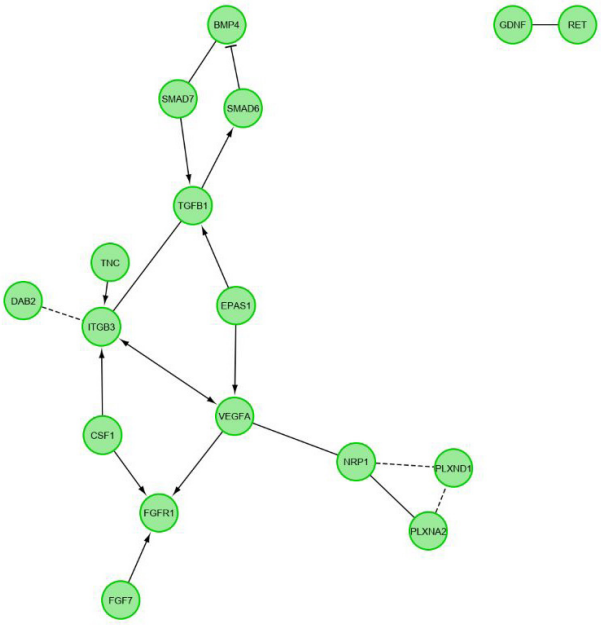


FIGURE 6 Functional interaction (FI) between differently expressed genes that belongs to the "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of a branching structure" and "morphogenesis of a branching epithelium" GO BP terms. In following figure "->" stands for activating/catalyzing, "-|" for inhibition, "-" for FIs extracted from complexes or inputs, and "---" for predicted FIs

Based on interaction visualization (**Fig. 5**) *BMP4* interacts with *FGFR1*, which is a receptor for fibroblast growth factors, that are involved in many processes, such as cell growth, maturation and division, angiogenesis and embryogenesis [25]. *FGFR1* expression gradually increased during *in vitro* culture, reaching the highest level on day 30; therefore it is another gene upregulated in OECs that is not specific for oviductal epithelium and is also expressed during many other developmental events. It has been reported, that *FGFR1* is crucial for correct neural tube development [26]. Pond et al. have shown that it is engaged in mammary gland branching morphogenesis [27]. As FGF signaling pathway is well known to be involved in tubular morphogenesis, increased expression of *FGFR1* during long-term OECs *in vitro* culture suggests that it also plays a role in epithelial tube development in porcine oviducts, and that the OECs maintain tube forming properties after long-term *in vitro* culture. Apart from that, this receptor's expression occurs e.g. in bovine corpus luteum [28] and porcine endometrial epithelial cells, as well as in stroma and glands [29].

TNC also exhibited increasing expression during the culture period, which may be due to the role of this protein in regulating cell adhesion. It also influences proliferation and interacts with fibronectin, integrin and growth factors. Tenascin C is known to be expressed in embryos, especially at regions of branching morphogenesis or epithelial-mesenchymal interactions, and in adults in stem cell niches and in inflammation sites [30]. Its upregulation may contribute to cell migration and tube formation, suggesting that *TNC* is another important factor engaged in oviductal development. Furthermore, Tenascin C may act via integrin *ITGB3* gene product, a subunit of a platelet membrane adhesive receptor complex GP IIb/IIIa, which also exhibited upregulation during our studies. *TNC* and *ITGB3* interaction has been described e.g. in breast cancer cells, where it resulted in FAK/Akt-473 pathway activation and cell migration [31]. In this case, such interaction may indicate tube forming activity in OECs, as integrin-mediated signaling pathway is known to be involved in epithelial apicobasal polarization – a crucial event in tubal development [6].

Apart from *ITGB3*-Tenascin C interaction, there is also a possible functional interaction between *ITGB3* and *DAB2* (Fig. 6.), expression of which is upregulated in OECs culture, reaching the highest level on day 30. *DAB2* is known to be involved in correct female reproductive tract functioning, as it is expressed in normal ovarian epithelial cells [32]. Hocevar et al. [33] demonstrated its important role in TGFβ signaling pathway, where it serves as a bridge between TGFβ receptors and Smad proteins and the study on bone marrow derived macrophages has shown that *DAB2* induces cell adhesion and spreading [34]. These results indicate this protein's important role in multiple signaling pathways and here we show its involvement in oviductal epithelium morphogenesis as well.

The transcription factors' role in OECs development also must be addressed, as they positively or negatively regulate many downstream genes' expression. For example, PAX2 is a transcription factor known to be involved in oviduct morphogenesis and is expressed in normal OECs [35]. However, we have observed a significant upregulation in other transcription factors' expression: *HHEX* and *EPAS1*, both engaged in endothelial cell development [36, 37].

HHEX is a transcription factor present in e.g. hematopoietic lineages [36], liver, thyroid and lungs [38]. In OECs long term *in vitro* culture it maintained relatively similar level on day 7, 15 and 30. This protein is known regulator of cell proliferation and differentiation – in prostatic and breast cancer cells its upregulation resulted in decreased cell migration by endoglin expression regulation [39]. *HHEX* is engaged in many developmental processes, including liver, heart, thyroid and pancreatic development [38] and our findings suggest that it may also be involved in oviductal morphogenesis. *EPAS1*, on the other hand, is a gene encoding HIF2A, a transcription factor involved in gene expression under hypoxic conditions [37] and we observed its highest level on day 30 of OEC's culture. *EPAS1* is expressed in highly vascularized tissues and endothelial cells, thus its role in vasculogenesis has been suggested [37]. However its involvement in oviduct development has not yet been reported.

We have also observed differential expression of enzyme-coding genes – *LOX* and *ARG1*, where *LOX*'s expression has risen gradually during *in vitro* culture and *ARG1* was upregulated on day 7. *LOX* initiates crosslinking of collagen and elastin and plays a role in stabilization of extracellular matrix [40]. Thus, it is expressed in tissues containing collagen or elastic fibers and is crucial for their stability. Apart from that, it binds with TGFβ1 and affects its signaling [41]. Therefore, it is an another component of multifunctional signaling pathway upregulated during this study. The study on gastric cancer cells indicates that *LOX* is involved in epithelial-mesenchymal transition under hypoxic conditions [42] and increases cellular proliferation, migration and invasion in endometrial epithelial cells [43]. Therefore, it is possible that it acts in a similar manner in OECs. The other aforementioned gene, *ARG1*, encodes arginase, which is well known to be involved in urea cycle and is expressed in the liver [44]. Apart from that, it had been demonstrated that its upregulation increases rat aortic smooth muscle cells proliferation [45]. Arginase I expression also occurs in highly regenerative mice tissues and provides necessary polyamine synthesis for effective development [46]. Therefore, we might suspect that it also affects initial steps of OEC morphogenesis.

SLIT proteins are known regulators of cell migration and apoptosis and their expression has already been reported in human and sheep ovaries [47], human endometrium and oviductal epithelial cells. It is suspected that these proteins negatively regulate a tubal embryo implantation, however there are further studies needed to test this hypothesis [48]. *SLIT2* connection with tubular development in ductal morphogenesis of mammary glands has already been investigated [49], which might be a reason why we have observed its increased expression on day 30 of OECs *in vitro* culture.

Taken together, our findings show that oviductal epithelial cells actively proliferate after long term *in vitro* culture. We have observed a significant increase in expression of genes belonging to "tube morphogenesis", "tube development", "morphogenesis of an epithelium", "morphogenesis of branching structure" and "morphogenesis of branching epithelium" GO BP terms, which suggests that OECs maintain their tube forming properties. The current knowledge on epithelial tubular development comes mostly from studies on MDCK kidney cells and breast MCF10A cells [6] and here we provide a novel insight into this process in porcine oviductal epithelial cells.

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

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