TRANSCRIPTOMIC PROFILE OF GENES ENCODING PROTEINS RESPONSIBLE FOR REGULATION OF CELLS DIFFERENTIATION AND NEUROGENESIS IN VIVO AND IN VITRO – AN OOCYTE MODEL APPROACH

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Abstract

The growth and development of the oocyte is essential for the ovarian follicle. Cumulus cells (CCs) - a population of granulosa cells - exchange metabolites, proteins and oocyte-derived paracrine factors with the oocyte through gap junctions, to contribute to the competency and health of the oocyte. This bi-directional communication of the cumulus-oocyte complex could be better understood through the micro-analysis of a porcine oocyte gene expression before *in vitro* maturation (IVM) and after. Additionally, the study of the somatic and gamete cells differentiation capability into neuronal lineage would be promising for future stem cell research as granulosa cells are easily accessible waste material from *in vitro* fertilization (IVF) procedures. Therefore, in this study, the oocytes of 45 pubertal Landrace gilts were isolated and the protein expression of the COCs were analyzed through micro-analysis techniques. Genes belonging to two ontological groups: neuron differentiation and negative regulation of cell differentiation have been identified which have roles in proliferation, migration and differentiation. Twenty identified porcine oocyte genes (*VEGFA*, *BTG2*, *MCOLN3*, *EGR2*, *TGFB3*, *GJA1*, *FST*, *CTNNA2*, *RTN4*, *MDGA1*, *KIT*, *RYK*, *NOTCH2*, *RORA*, *SMAD4*, *ITGB1*, *SEMA5A*, *SMARCA1*, *WWTR1* and *APP*) were found to be down-regulated after the transition of IVM compared to *in vitro*. These results could be applied as gene markers for the proliferation, migration and differentiation occurring in the bi-directional communication between the oocyte and CCs.

Running title: Differentiation and neurogenesis in oocyte cells

Keywords: pig, oocyte, cumulus-oocyte complex, microarray

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Introduction

The growth and development immature female egg cell -called the oocyte – is a complex process involving the varied expression of many proteins, metabolites and environment [1]. Our understanding of the mechanisms surrounding fertilization and *in vitro* fertilization (IVF) would benefit from more information on the oocyte such as potential biomarkers, stages of its growth, and what genes could potentially improve the competency of these cells.

The oocyte sits in the center of the ovarian follicle, surrounded by layers of granulosa cells and then thecal cells [2]. The granulosa cells are split in two populations: cumulus cells (CCs) and mural granulosa cells (GCs). To improve fertility and health of the follicle, interest is increasingly surrounding the bi-directional communication between the oocyte and CCs, a relationship identified as the cumulus-oocyte complex (COC).

The state of the cumulus-oocyte complex directly impacts the health and competence of the oocyte by cell nuclear regulation of meiosis [3]. The bi-directional communication between the gamete and somatic cell populations are conducted through gap junctions made up of connexins, and there is numerous evidence of molecules being exchanged to regulate the other's activity such as proteins, oocyte-derived paracrine factors or glucose metabolic enzymes [4,5]. Poor fertility is often associated with a deficiency in communication from the CC side. Despite this co-operative relationship, not much is known about the gene expression of the COC.

Micro-array analysis and RT-qPCR can be used to study the transcriptome of the oocyte during its development, and identify molecular markers involved in neuron differentiation and negative regulation of cell differentiation [6]. In addition to building our understanding of the maturation of the ovarian follicle, these possible molecular markers could be used to try and manipulate CCs for their possible stemness qualities in a similar manner as Kossowska-Tomaszczuk's lab were able to expose *in vitro* GCs differential capabilities [7].

In this study, the protein expression of genes belonging to 'neuron differentiation' and 'negative regulation of cell differentiation' will be chosen and analyzed *in vitro* and IVM of a porcine oocyte model. We aim to find if the genes are up- or down-regulated after IVM has taken place to better understand the microenvironment of the COC and establish the possible implications of the somatic and gamete cell communications.

Material and methods

Experimental design

Oocytes were collected and subjected to two Brilliant Cresyl Blue (BCB) tests and divided into two groups. The first group ("before IVM") included oocytes graded as BCB-positive (BCB⁺) and directly ex-

posed to microarray assay. The second group ("after IVM") included BCB⁺ oocytes which were then *in vitro* matured, and if classified as BCB⁺ in second BCB test passed to molecular analyses.

Animals

A total of 45 pubertal crossbred Landrace gilts bred on a commercial local 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 animals were bred under the same conditions and fed the same forage (depending on age and reproductive status). All experiments were approved by the Local Ethic Committee (approval no. 32/2012).

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

The ovaries and reproductive tracts were recovered at slaughter and transported to the laboratory within 40 min. at 38°C in 0.9% NaCl. To provide optimal conditions for subsequent oocyte maturation and fertilization *in vitro*, the ovaries of each animal were placed in a 5% fetal bovine serum solution (FBS; Sigma-Aldrich Co., St. Louis, MO, USA) in PBS. Single large follicles (>5mm) were opened by puncturing with a 5ml 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 gentamycin, and 0.5 mg/ml BSA (Sigma-Aldrich, St. Louis, MO, USA). The COCs were selected under an inverted microscope Zeiss, Axiovert 35 (Lübeck, Germany), counted, and morphologically evaluated. Only COCs of grade I possessing homogeneous ooplasm and uniform, compact cumulus cells were considered for further use, resulting in a total of 300 grade I oocytes (3 x n=50 "before IVM" group, 3 x n=50 "after IVM" group).

Assessment of oocyte developmental competence by BCB test

Brilliant Cresyl Blue (BCB) test was used for assessment of porcine oocytes' quality and maturity. The glucose-6-phosphate (G6PDH) enzyme converts BCB stain from blue to colorless. In oocytes that completed the growth activity of the enzyme decreases and the stain cannot be reduced, resulting in blue oocytes (BCB⁺). To perform the BCB staining test, oocytes were washed twice in modified Dulbecco's Phosphate Buffered Saline (DPBS) commercially supplemented with 0.9 mM calcium, 0.49 mM magnesium, 0.33 mM pyruvate, and 5.5 mM glucose (Sigma-Aldrich, St. Louis, MO, USA), and additionally with 50 IU/ml penicillin, 50 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA), and 0.4% Bovine Serum Albumin (BSA) [w/v] (Sigma-Aldrich, St. Louis, MO, USA). They were then treated with 13 µM BCB (Sigma-Aldrich, St. Louis, MO) diluted in

DPBS at 38.5°C, 5% CO₂ for 90 min. After treatment, the oocytes were transferred to DPBS and washed twice. During washing, the oocytes were examined under an inverted microscope and classified as stained blue (BCB⁺) or colorless (BCB⁻). Only the granulosa cell-free BCB⁺ oocytes were used for subsequent molecular analyses ("before IVM" group) or IVM followed by second BCB test and molecular analyses ("after IVM" group).

In vitro maturation of porcine cumulus-oocyte-complexes (COCs)

After the first BCB test, the BCB⁺ COCs were subjected to IVM. The COCs were cultured in Nunclon™Δ 4-well dishes (Thermo Fisher Scientific, Waltham, MA, USA) in 500 µl 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 (NacalaiTesque, 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% (v/v) filtered porcine follicular fluid, and gonadotropin supplements at final concentrations of 2.5 IU/ml hCG (human Chorionic Gonadotropin) (Ayerst Laboratories, Inc., Philadelphia, PA, USA) and 2.5 IU/ml eCG (equine Chorionic Gonadotropin) (Intervet, Whitby, ON, Canada). Wells were covered with mineral oil overlay and cultured at 38°C under 5% CO₂ in air for 22h, and then for additional 22h in medium without hormones. After cultivation, the second BCB staining test was performed, and BCB⁺ oocytes were used for further molecular analyses.

Microarray expression analysis and statistics

Experiments were performed in three replicates. Total RNA (100 ng) from each pooled sample was subjected to two round 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 Affymetrix® Porcine Gene 1.1 ST Array Strip (48°C/20 h). Then, microarrays were washed and stained according to the technical protocol, using Affymetrix GeneAtlas Fluidics Station. The array strips were scanned employing Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. Quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis software.

All analyzes were performed using BioConductor software, based on the statistical R programming

language. For background correction, normalization and summation of raw data, the Robust Multitarray Averaging (RMA) algorithm implemented in "affy" package of BioConductor was applied. Biological annotation was taken from BioConductor "oligo" package where annotated data frame object was merged with normalized data set, leading to a complete gene data table. Statistical significance of analyzed genes was performed by moderated t-statistics from the empirical Bayes method. Obtained p value was corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on p value beneath 0.05 and expression fold higher than |2|.

Functional annotation clustering of differentially expressed genes was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery). Gene symbols for up- or down-regulated genes from each of the compared groups were loaded to DAVID by "RDAVIDWebService" BioConductor package. For further analysis we have chosen the enriched GO terms which has at least 5 genes and p-value (Benjamini) lower than 0.05. The enriched GO terms were subjected to hierarchical clusterization algorithm and presented as a heat maps.

Subsequently we analyzed the relation between the genes belonging to chosen GO terms with GOplot package. 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 estimating the change course of each gene-ontology term.

Interactions between chosen differentially expressed genes/proteins belonging to ontology group were investigated by STRING10 software (Search Tool for the Retrieval of Interacting Genes). List of gene names were used as query for interaction prediction. Searching criteria based on co-occurrences of genes/proteins in scientific texts (text mining), co-expression and experimentally observed interactions. The results of such analysis generated gene/protein interaction network where the intensity of the edges reflects the strength of interaction score. Besides interaction prediction, STRING also allowed us to perform functional enrichments of GO terms based on previously uploaded gene sets.

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 from 30.06.2012.

Results

Whole transcriptome profiling by Affymetrix microarray allowed us to analyze the gene expression changes in freshly isolated oocytes, before *in*

FIGURE 1 Heat map representations of differentially expressed genes belonging to the “neuron differentiation” and “negative regulation of cell differentiation” GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colours (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, Entrez gene IDs, ratio and corrected P values of studied genes

GENE SYMBOL	ENTREZ GENE ID	RATIO	P VALUE
VEGFA	397157	-14.34938689	0.001912689
BTG2	100048932	-13.44331893	0.0000955
MCOLN3	100625693	-7.203754451	0.000850265
EGR2	100038004	-6.042156175	0.007949861
TGFB3	397512	-5.088482493	0.000405979
GJA1	100518636	-4.833081147	0.000107676
FST	445002	-4.450446449	0.000364693
CTNNA2	100525337	-4.349239665	0.000512181
RTN4	100170118	-4.321937118	0.027495815
MDGA1	397529	-4.293184001	0.003493167
SLITRK3	106504067	-4.181617506	0.004260951
INHBA	397093	-4.144909849	0.000148036
CDK6	100518921	-4.032235086	0.006042481
LAMB2	101101688	-3.586055237	0.000187911
ROBO2	100517681	-3.524243185	0.001183495
CUX1	100521258	-3.342052921	0.000372663
IHH	397174	-3.278733207	0.000551261
EMX2	100152562	-3.12387706	0.001929388
ZCCHC11	100516979	-3.109237139	0.019809962
APP	397663	-3.08509997	0.005602323
WWTR1	100522573	-3.056215175	0.000254025
SMARCA1	100188905	-3.038211613	0.014758847
SEMA5A	100737194	-2.829721119	0.001092396
ITGB1	397019	-2.73050204	0.003705215
SMAD4	397142	-2.71885268	0.001238681
RORA	100156637	-2.60467925	0.021553766
NOTCH2	100153369	-2.598575668	0.002523723
RYK	100523513	-2.370213023	0.00439989
KIT	396810	-2.323181414	0.00255635

vitro procedure (“before IVM”), in relation to after *in vitro* maturation (“after IVM”). By Affymetrix® Porcine Gene 1.1 ST Array we have examined expression of 12258 porcine transcripts. Genes with fold change higher than |2| and with corrected p value lower than 0.05 were considered as differentially expressed. This set of genes consisted of 419 different transcripts. Subsequently, the genes were used for identification of significantly enriched GO BP terms.

DAVID (Database for Annotation, Visualization and Integrated Discovery) software was used for extraction of the genes belonging to regulation of “neuron differentiation” and “negative regulation of cell differentiation” gene ontology Biological Process term (GO BP). We found that 29 genes from these GO BP term were significantly represented in down-regulated gene sets. This set of genes was subjected to hierarchical clusterization procedure and presented as heat maps (Fig. 1).

Set of the differentially expressed genes belonging to “neuron differentiation” and “negative regulation of cell differentiation” GO BP terms with their official gene symbols, Entrez Gene IDs, ratio and corrected p values were shown in table 1.

The enrichment of each GO BP term as well KEGG pathway were calculated as z-score and shown on the circle diagram (Fig. 2)

Moreover, in Gene Ontology database genes that formed one particular GO group can also belong to other different GO term categories. By 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-generated interaction network was created with differentially expressed genes belonging

to the “fatty acid metabolic process” ontology group. The intensity of the edges reflects the strength of interaction score (Fig. 5).

Discussion

The cumulus-oocyte complex (COC) describes the barrier between cumulus cells (CCs) and the oocyte they surround [8]. Numerous hormonal secretions and changes in protein expression occur throughout growth and maturation of the oocyte, which makes up the inner layer of the ovarian follicle. CCs (with the higher metabolic activity) deliver energy and proteins necessary for the oocyte via gap junctions, and as the oocyte matures: gene expression, organelle distribution and the proteins present will be altered [8]. CCs are one of two populations of granulosa cells, the other being mural granulosa cells (GCs) which make up the outer layers of an ovarian follicle [9]. The essential processes for the growth and maturation of the ovarian follicle, called oogenesis and folliculogenesis, are controlled by granulosa cell and theca cell function [10]. GCs and CCs are waste material from IVF and are therefore accessible cells for studies [11].

To better understand how the growth and development of the ovarian follicle, studying the relationship between oocytes and CCs is important. One way to do this is by comparing the change of gene expression in an oocyte model before *in vitro* maturation (IVM) and after. Due to CCs involvement in oocytes *in vitro* growth and *in vitro* maturation (IVM), further study of the COC could shed light on the oocytes potential to differentiate into neuronal cells.

In this study, the expression of a group of marker genes in ontology groups ‘neuron differentiation’ and ‘negative regulation of cell differentiation’ were

FIGURE 2 The circle plot showing the differentially expressed genes and z-score “neuron differentiation” and “negative regulation of cell differentiation” GO BP Terms. The outer circle shows a scatter plot for each term of the fold change of the assigned genes. Red circles display downregulation. 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 differently expressed genes that belong to the “neuron differentiation” and “negative regulation of cell differentiation” GO BP Terms. The ribbons indicate which gene belongs to which categories. The middle circle represents logarithm from fold change (LogFC) between before IVM and after IVM respectively. The genes were sorted by logFC from most to least changed gene

examined before and after IVM of the oocyte to evaluate the cell model’s stemness. After IVM, we found 29 down-regulated genes belonging to our chosen ontology groups, which can be used to suggest the change in oocyte function in this stage of development and how the oocyte interacts with CCs. The 10 most negatively regulated genes were selected and analyzed: *VEGFA*, *BTG2*, *MCOLN3*, *EGR2*, *TGFB3*, *GJA1*, *FST*, *CTNNA2*, *RTN4* and *MDGA1*.

VEGFA was the most downregulated selected and analyzed gene after IVM, and belongs to the ‘neuron differentiation’ ontology group. *VEGFA* plays an important role in inducing vascularization and angiogenesis, and its capability is emphasized by being able to induce neo-angiogenesis single-handedly

[12]. The dramatic change in expression of the gene after IVM indicates an importance in regulating the development and maturation of the oocyte. Gao et al.’s study supports that *VEGFA* is down-regulated in oocytes after IVM [13]. *VEGFA* inhibits *SMAD4*, catalyzes *KIT* and *ITGB1*, and also binds to the latter gene.

The second most down-regulated gene was found to be *BTG2* which regulates progenitor cell numbers and differentiation [14]. It will only act to induce differentiation of progenitor cells to orthosympathetic neurons in the presence of *NGF*. A study done by Li et al. suggest that the BTG family that *BTG2* is a member of, has an essential role in regulating the cell cycle of granulosa and thecal cells [15]. If overexpression of the gene arrests the cell cycle,

FIGURE 4 Heatmap showing the gene occurrence between differently expressed genes that belongs to the “neuron differentiation” and “negative regulation of cell differentiation” GO BP Terms. The intensity of the color is corresponding to amount of GO BP terms that each gene belongs to

FIGURE 5 STRING-generated interaction network between genes that belongs to the “neuron differentiation” and “negative regulation of cell differentiation” GO BP Terms. The intensity of the edges reflects the strength of interaction score

then this would explain why the maturation of the oocyte would downregulate this gene.

The next most negatively regulated gene that we selected and analyzed is MUCOLIPIN-3 (*MCOLN3*),

which is a member of the mucolipin superfamily of transient receptor potential (TRP) channels [16]. The protein localizes to intracellular vesicles, in addition to the plasma membrane, and is involved in

the endosomal pathway for calcium ion transport [17]. Found in both granulosa cells and ovaries, there is evidence that the gene helps drive differentiation in at least hair cells [18], in addition to being part of our 'neuron differentiation' ontology group.

EGR2 (early growth receptor 2) is the fourth most negatively expressed gene, and is necessary for peripheral myelin production [19]. This is shown by the process of myelination and nerve injury triggering the same expression profile of *EGR2* protein [20]. Furthermore, *EGR2* deficient mice did not express other genes that are associated with myelin [21]. Jin et al. have hypothesized that *EGR2* is crucial for granulosa cells, as it interacts with gonadotropins to during folliculogenesis [22]. However, after IVM occurs, the expression of the gene has perhaps a less necessary function in the oocytes, which is supported by Brązert et al.'s study [23].

The fifth most negatively regulated gene is the first one from the 'negative regulation of cell differentiation' ontology group, and is *TGFBR3*. The activities of transforming growth factor-beta (TGFβ) and inhibin are modulated by *TGFBR3*. In the presence of the receptor, TGFβ will more readily bind to *TGFBR2*, and also inhibin more strongly repels the activity of activin and Bone Morphogenetic Proteins (BMPs). *TGFBR3* null mice resulted in liver and heart defects, which suggestively led to neonatal deaths [24]. Therefore, its expression would be essential for any endeavors to guide neurogenic differentiation in oocyte cells. *TGFBR3*'s change in expression in porcine oocytes before and after IVM has been examined before in Kranc et al.'s microanalysis study [25].

GJA1 (Gap Junction protein, alpha-1) is the sixth most negatively expressed gene and is part of the 'neuron differentiation' ontology group. Human connexin 43 (Cx43) is encoded by *GJA1* [26]. Being responsible for cell-cell communication throughout the human organ systems, mutations in *GJA1* are connected with ischemia and heart failure. Furthermore, negative neuronal differentiation and signaling is strongly linked to the protein's activity [27]. In the ovaries, *GJA1* form gap junctions for the COC so the microenvironment can communicate, and the ovaries of women who have PCOS show a lower expression of *GJA1* [28].

FST (follistatin) is the seventh most downregulated gene, and the second in the chosen 'negative regulation of cell differentiation' ontology group. Follicle stimulating hormone is believed to be regulated by *FST* which inhibits the hormones activity [29]. Activin and inhibin, which negatively regulate each other, are also affected by *FST*, and downregulates activin's activity in differentiating granulosa cells [30]. Multiple studies support that *FST* expression strongly affects the development and vitality of the oocyte and even the COC significantly [31–33]. In our study, we have evidence that *FST* binds with *INHBA*.

CTNNA2 (catenin cadherin-associated protein, alpha 2) is the eighth most downregulated gene, and is involved in 'neuron differentiation'. The protein encoded is found to be mostly expressed in the central nervous system [34]. The absence of *CTNNA2* results in problems for neuronal processes and cell migration [35]. Budna et al. also studied the expression of *CTNNA2* in oocytes (originating from cows) before and after IVM, and related the gene to function as cell recognition and adherence [36]. They did not find any statistical significance in the change of expression between the two time periods, unlike in this study.

Nogo proteins are encoded by *RTN4* (reticulon-4), which is the ninth most downregulated gene and also in our 'neuron differentiation' ontology group. These proteins have myelin-associated functions important for the endoplasmic reticulum proteins [37]. These functions include cell migration, metastasis, death and adhesion for myocytes, endothelial cells, and neurons. In studies of ischemic animal models, it has been suggested that the expression of *RTN4* will increase to protect nerve function against the stress to a certain extent before the overexpression causes apoptosis of non-neurons [38]. In the study of Kranc et al., it was also noted *RTN4* was less upregulated after IVM compared to before [39].

Our final gene is the ten most downregulated genes after IVM is *MDGA1* (MAM domain containing glycosylphosphatidylinositol anchor 1 gene). Included in the 'neuron differentiation' group, *MDGA1* is necessary for the migration of neural cells, such as to adhere to the top layer of cortical plates [40], and its expression can be used to detect neurons ReIn-positive Cajal and Retzius [41]. There are little studies on *MDGA1*'s effect on oocyte or cumulus cells.

The least downregulated genes from our chosen GO BP terms were *KIT*, *RYK*, *NOTCH2*, *RORA*, *SMAD4*, *ITGB1*, *SEMA5A*, *SMARCA1*, *WWTR1* and *APP*.

KIT, *ITGB1*, *NOTCH2* and *WWTR1* all belonged to the 'negative regulation of cell differentiation' ontology group. All of their functions contribute to the growth and development of the oocyte and would explain their higher expression to the other genes in our study. As mentioned before, our studies believe both *KIT* and *ITGB1* catalyze *VEGFA* activity. In a follicle, *KIT* ligand prevents apoptosis of preantral cells so they can transition to the antral follicle stage [42], and, like *VEGFA*, its expression promotes proliferation of granulosa cells and the oocyte [43]. *ITGB1* also furthers the development of the follicle in folliculogenesis, being involved in embryonic processes such as trophoblastic implantation [36]. Evidence from knockout mice studies and the association of the genes expression with PCOS, it is suggested that formation of the primordial follicle also relies on the expression of *NOTCH2* (a member of the *NOTCH* family) in granulosa cells [44]. *WWTR1* is a target gene in the Hippo pathway, which phos-

phorylates the protein to increase its promotional activity of proliferation and maturation of granulosa cells during ovulation [45].

Interestingly, most of the higher regulated genes are part of our 'neuron differentiation' GO BP terms group. These genes are *RYK*, *RORA*, *SMAD4*, *SEMA5A*, *SMARCA1* and *APP*. One of the receptors for Wnt is receptor-like tyrosine kinase (*RYK*), and consequently acts to promote neurogenesis and axonal growth [46]. A study done by Budna et al. supports that expression of *RYK* is downregulated after IVM [47], and its role in follicular development should be further studied. Related morphan receptor A (*RORA*) is usually found in mesenchymal stem cells originating from bone marrow, and its function in the oocyte could be regulating its development [48]. Kawaguchi-Niida et al. have shown that, for the TGF β /BMP signaling pathway, expression of *SMAD4* is needed to ensure differentiated neural progenitor cells don't reverse their transition [49]. The presence of *SMAD4* was also studied by Xing et al., and in particular they found it mainly expressed in the oocyte, but also in its surrounding granulosa and theca cells, whilst its expressing being dependent on the stage of follicle development [50].

SEMA5A is a member of the semaphoring family and expressed by oligodendrocytes and their progenitor cells [51]; and its expression was also found in oocytes to be increased in Nawrocki et al.'s study comparing gene expression *in vitro* compared to *in vitro* [52]. *SMARCA1* encodes a protein which regulates chromatin remodeling, and inhibiting its function increased brain size in mice [53]. Finally, our last selected and analyzed gene is amyloid precursor protein (*APP*). Neuronal and brain development is influenced by the protein, the former relying on the APPs cleavage in precursor and neuron cells [54].

The genes belonging to our 'neuron differentiation' ontology group term have relatively little knowledge on how they affect processes concerning the oocyte, and even less on its interaction with cumulus cells. Therefore, more research should be done on these noted genes as insight could be given from understanding the COC microenvironment. With these results, our analyzed genes could perhaps be used for the purpose oocyte maturation biomarkers.

Conclusions

To summarize, we have identified the downregulation of various genes after the transition from *in vitro* to *in vitro* maturation through an oocyte model. The identified genes were part of two chosen ontological groups: 'negative regulation of differentiation' or 'neuron differentiation', with roles in the microenvironment such as proliferation, migration and differentiation. Our findings would like to highlight that the expression of the genes occurred due to the cumulus cells and the cellular relationship

between the oocytes. Rather than a merely an exchange of nutrients, the COC provides the proteins and cellular environment necessary for processes such as folliculogenesis and oogenesis. Therefore, in future work, further focus should be given to the COC in order to improve the competency and understanding of the development of the follicle. Stemness of the cumulus cells and oocyte should also be considered because of our ontology groups expression, as differentiation could be directed in future studies.

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Conflict of interest statement

The authors declare they have no conflict of interest.

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