NEW MARKERS OF HUMAN CUMULUS OOPHORUS CELLS CULTURED IN VITRO – TRANSCRIPTOMIC PROFILE

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Abstract

The presence of CCs around the oocyte after ovulation is one of the key elements contributing to oocyte developmental competence. In the presented study, we used CCs from 12 patients aged 18-40 diagnosed with infertility. After harvesting cells on day 1, 7, 15 and 30 of culture, total RNA was isolated and transcriptomic analysis was performed. The DAVID software indicated the following GO BP terms: “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility”. Of the genes belonging to all ontological groups, the most downregulated were: SLC7A8, DFNB31, COL1A1, CDC42SE1, TGFBR3, HMGB1, with the most upregulated genes being: ANXA3, KIAA1199, HTR2B, VCAM1, DKK1.

While many studies focus on attempts to obtain fully competent oocytes, scientists still have difficulty attaining adequate results *in vitro*. Lack of adequate knowledge often results in low *in vitro* fertilization efficiency. Therefore, our research focuses on CCs cells, thanks to which the oocyte most likely acquires developmental competence. The main purpose of the study was to identify the potential molecular markers responsible for cell junction organization, migration, differentiation, morphogenesis and motility.

Running title: New markers of human cumulus oophorus cells cultured *in vitro*

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Introduction

Cumulus cells (CCs) differ significantly from granulosa cells (GCs), resting on the basal lamina of ovarian follicle. CCs are in close physical contact with the oocyte, forming a cumulus-oocyte complex (COC). The oocyte controls the differentiation and expansion of CCs, which in turn are involved in the metabolism of pyruvate and glucose consumed during energy production in the oocyte [1,2]. Due to this proximity, CCs are influenced by regulatory factors produced by the oocyte. These regulatory factors include primarily: growth differentiation factor 9 (GDF9), bone morphogenetic protein 15 (BMP15) and fibroblast growth factor 8 (FGF8). They are primarily responsible for suppressing expression of the LH receptor and genes responsible for steroid production. Unlike the GCs, which “remain” in the ruptured follicle, CCs are “ejected” from the ruptured follicle during ovulation [3]. CCs also produce many inflammatory factors and cytokines that are released during ovulation. Cumulus cell-oocyte complex (COC) released from the ovary is therefore a kind of a microenvironment [4–6]. Increased concentration of gonadotropins in the female body causes increased production of hyaluronic acid by CCs, which expands the spaces between these cells. Correct ovulation requires the production of prostaglandins. GDF9 (a member of the TGF- β superfamily) plays a major role in the induction of Ptg2 expression in CCs through increased luteinizing hormone (LH) concentration. Lack of GDF9 blocks the development and growth of follicles, thus leading to infertility [7].

In most mammal species, including humans, CCs cells surround the oocyte at conception. It is believed that CCs interact with the oocyte and sperm, and thus are involved in promoting the fertilization process and oocyte developmental competence [8,9]. It has been proven many years ago that CCs are involved in maintaining myocyte retention in the oocyte. Oocytes removed from the follicle, lacking CCs, resume meiotic division, which should be completed only after fertilization [10]. In addition, CCs facilitate the capture of COC by ciliated oviduct cells [11]. Glycodelin-C, a derivative of glycodelin, is isolated from the matrix of CCs cells. This substance stimulates the binding of spermatozoa to the zona pellucida of oocyte [9]. It has also been reported that the rate of CCs associated with morphologically abnormal oocytes or immature oocytes is much higher than in CCs surrounding morphologically normal, mature oocytes. The increase in CC apoptosis has a negative impact on a number of processes related to the correct fertilization and development of blastocyst and reduces the effectiveness of *in vitro* fertilization (IVF) [12,13].

The expression of genes associated with the proper function of CCs results from a number of factors produced by the oocyte, but also the environ-

ment of the mature ovarian follicle, and all processes occurring during follicle growth in the ovarian microenvironment [14].

CCs also produce antioxidant compounds that reduce the level of oxidative stress caused by reactive oxygen species (ROS). These compounds, mainly superoxide dismutase (SOD) [15] and Glutathione transferase S theta 1 (GSTT1) protect the oocyte against oxidative stress [16,17].

Knowledge about the expression of individual genes, as well as the presence of individual components of the intracellular metabolic pathways, or the CCs apoptosis index can become a source of valuable molecular markers determining the developmental competence of oocytes. It is known that the expression of amphiregulin (AREG) and epiregulin (EREG) (epidermal growth factor (EGF) -like factors) depends on the dose of LH administered during ovarian stimulation. Therefore, it is suggested that AREG and EREG are part of the signal transduction pathway, which leads to the release of the oocyte from the mature ovarian follicle and the luteinization process in women [18]. Recent studies described of new properties of CCs. CCs produce a large amount of hyaluronan, which targets CD44 (marker of cancer cells). Culture of pancreatic cancer cells in a medium conditioned with CCs activated pro-apoptotic genes in these cancer cells [19]. The mechanisms of communication between the oocyte and CCs cells through gap connections are well known. It is known, however, that this communication also takes place by means of paracrine signaling. Less known methods of communication are the transfer of non-coding RNAs through exosomes from the cumulus to the oocyte [20]. Coenzyme Q10 (CoQ) is another factor that has a significant impact on the quality of oocyte and CCs cells. This relationship is important for the proper functioning of mitochondria, not only in the oocyte but also in CCs cells. A decrease in mitochondrial activity is associated with aging of oocytes. Therefore, reduced CoQ production in older women translates into reduced fertility and a higher risk of birth defects for embryos [21].

Understanding the molecular mechanisms and regulation of gene expression in CCs may reflect processes in the oocyte. Changes occurring at the molecular level may be a signpost for identifying oocyte quality, oocyte acquisition of developmental competences, and quality of obtained blastocyst. The main purpose of the research was to identify the potential molecular markers responsible for cell junction organization, migration, differentiation, morphogenesis and motility.

Material & Methods

Patients

CCs were obtained from patients undergoing IVF. The study used CCs from 12 patients aged 18–40 diagnosed with infertility. The factors excluding

patients from these studies were: polycystic ovary syndrome (PCOS), AMH less than 0.7 ng/ml, antral follicle count less than 9, day 2-3 FSH serum level higher than 15 mU/ml and endometriosis. All *in vitro* fertilization procedures were conducted in the Department of Infertility and Reproductive Endocrinology, Poznan Medical University, Poland.

The IVF procedure was based on adapted and controlled ovarian hyperstimulation protocol. FSH (Gonal-F, Merck Serono) and highly purified hMG-HP (Menopur, Ferring) were used for ovarian stimulation. Additionally, to stop pituitary functions, Cetrorelix Acetate (Cetrotide, Merck Serono) injections at the right dose were performed. Ovulation was induced by injection of 6500 U hCG (Ovitrelle, Merck-Serono).

After oocyte pick-up (OPU), oocyte-cumulus complexes (COCs) have been selected by an embryologist for further IVF procedure. In the next step of IVF routine, the obtained COCs were denuded. Corona radiata cells and cumulus oophorus somatic cells forming COCs have been removed during denudation process (800 IU/mL of HYASE-10X). CCs obtained in this way from multiple follicles of one patient were pooled and transferred to cell culture laboratory for further analysis.

Condition of long-term primary *in vitro* culture

CCs were collected after denudation of oocytes. Afterwards, they were washed twice with basal culture medium and centrifuged at RT (200 x g for 10 min). Basal culture medium consisted of DMEM (Dulbecco's Modified Eagle's Medium, Sigma; Merck KGaA, Darmstadt, Germany) supplemented with 10 mg/ml gentamicin (Invitrogen; Thermo Fisher Scientific, Inc.), 2% fetal bovine serum FBS (FBS; Sigma; Merck KGaA), 4 mM L-glutamine (stock 200 mM, Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10,000 U/ml penicillin and 10,000 µg/ml streptomycin (Invitrogen; Thermo Fisher Scientific, Inc.) [22].

CCs were cultured at 37°C in 5% CO₂ and humid atmosphere. After attaining 90% confluence, the cells in the culture were detached from the bottom of the 6-well plate by 1-2 min incubation with 0.05% trypsin-EDTA (Invitrogen; Thermo Fisher Scientific, Inc.). Later, the cells were counted using the ADAM Cell Counter and Viability Analyzer (Bulldog Bio). CCs were cultured for 30 days. Medium was changed every 72-75 hours of culture. Cells for the analysis were harvested on day 1, 7, 15 and 30 of *in vitro* culture.

Total RNA isolation

After harvesting cells on the 1st, 7th, 15th and 30th day of culture, total RNA was isolated. The process of RNA isolation was performed according to modified method of Chomczyński and Sacchi [23]. Briefly, obtained CCs were suspended in 1 ml of monophasic guanidine thiocyanate and phenol solution (TRI Re-

agent®, Sigma; Merck KGaA). Next, chloroform was added to separate the phases during centrifugation. The upper aqueous phase, containing isolated RNA, was collected. RNA was extracted with 2-propanol (Sigma; Merck KGaA, catalog number I9516), added in an amount adequate for 1 ml of TRI-reagent. Finally, RNA was washed with 75% ethanol, dried, resuspended in 20 µl of pure water and measured.

Microarray expression analysis and statistics

Total RNA (100ng) was converted to double-stranded cDNA. In the next step, labeled complementary RNA (cRNA) was synthesized and amplified by *in vitro* transcription of the double-stranded cDNA template (GeneChip™ 3'IVT PLUS Reagent Kit, Applied Biosystems™, Foster City, CA, USA). Obtained cRNA was fragmented by divalent cations and elevated temperature. Fragmented and labeled cRNA (7.5 µg) were hybridized to Human Genome U219 Array Strip (45°C/16 h, Applied Biosystems™, Foster City, CA, USA). Then, the microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by Imaging Station of the GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ Operating Software. The 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 of presented analyses and graphs were performed by Bioconductor and R programming language. 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.

Statistical significance of the 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. Differentially expressed genes were subjected to the selection of genes involved in cellular morphogenesis, junction and migration. Differentially expressed gene list was uploaded to the DAVID software (Database for Annotation, Visualization and Integrated Discovery), where "cell junction organization", "cell migration", "cell morphogenesis involved in differentiation", "cell morphogenesis" and "cell motility" GO BP terms were obtained. Expression data of these genes were subjected to hierarchical clustering procedure and presented as a heatmap graph. Detailed analysis of genes belonging to selected GO BP terms were presented as plots using "GOplot" library [24].

Moreover, the list of differentially expressed genes from selected GO BP terms was uploaded to the

FIGURE 1 Heatmaps presenting differentially expressed genes involved in “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility” based on GO BP terms. Each row on the Y axis represents a single transcript. The red color indicates downregulated genes while the green are upregulated

STRING software (Search Tool for Retrieval of Interacting Genes/Proteins) for interaction prediction.

Finally, we used ReactomeFIViz app from the Cytoscape software for creating the Reactome Functional Interaction (FI) network from the set of differentially expressed genes.

Ethical approval

This research has been approved by Poznań University of Medical Sciences Bioethical Committee with 1290/18 resolution.

Results

We used Human Genome U219 Array Strip for the microarray gene expression analysis of human

cumulus oophorus cells. This method allowed us to study the gene expression of 22,480 transcripts at 1, 7, 15 and 30 days of *in vitro* cumulus oophorus cell culture. We selected genes with more than 2-fold changes and corrected p-values less than 0.05 for downstream analysis. A total of 4773 differentially expressed genes (DEGs) were identified according to the above criteria. We started the microarray gene expression analysis with subjecting the list of DEGs to DAVID software, which showed that the genes can be assigned to 775 GO BP, 33 GO MF and 125 GO CC gene ontology terms. This paper focused on the genes involved cellular morphogenesis, junction and migration. The DAVID software indicated the following GO BP terms, which cover the

TABLE 1 The 10 most significantly upregulated and all of the downregulated genes involved cellular morphogenesis, junction and migration

Gene symbol	Gene name	Fold change	Adj. p.val
DKK1	dickkopf WNT signaling pathway inhibitor 1	34.81	<0.05
ANXA3	annexin A3	34.27	<0.05
KIAA1199	KIAA1199	27.73	<0.05
VCAM1	vascular cell adhesion molecule 1	26.69	<0.05
HTR2B	5-hydroxytryptamine (serotonin) receptor 2B, G protein-coupled	24.33	<0.05
CTGF	connective tissue growth factor	18.97	<0.05
TGFBR2	transforming growth factor, beta receptor II (70/80kDa)	15.71	<0.05
STC1	stanniocalcin 1	14.96	<0.05
CD74	CD74 molecule, major histocompatibility complex, class II invariant chain	14.18	<0.05
SEMA5A	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	13.15	<0.05
SLC7A8	solute carrier family 7 (amino acid transporter light chain, L system), member 8	-2.00	<0.05
DFNB31	deafness, autosomal recessive 31	-2.01	<0.05
COL1A1	collagen, type I, alpha 1	-2.03	<0.05
CDC42SE1	CDC42 small effector 1	-2.04	<0.05
TGFBR3	transforming growth factor, beta receptor III	-2.05	<0.05
HMGB1	high mobility group box 1	-2.05	<0.05

FIGURE 2 The circular scatter plots of differentially expressed genes involved in “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility” GO BP terms. Each dot represents a single gene. The z-scores were presented as segments of inner circles

above processes: “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility”. The 150 genes involved in those processes were clustered using hierarchical clustering and presented as heat-maps (**Fig. 1**). It is worth mentioning that 144 genes were upregulated, which is the greater part of the list of genes used for hierarchical clustering. The down-regulated genes are: *SLC7A8*- solute carrier family 7 (amino acid transporter light chain, L system), member 8; *DFNB31*- deafness, autosomal recessive 31; *COL1A1*- collagen, type I, alpha 1; *CDC42SE1*- CDC42 small effector 1; *TGFBR3*- transforming growth factor, beta receptor III; *HMGB1*- high mobility group box 1. The direction of expression change (upregulation or downregulation) was maintained in cumulus oophorus cell culture in subsequent points of analy-

sis (after 7, 15 and 30 days of *in vitro* culture). The 10 most significantly upregulated and all of the down-regulated genes, their symbols, fold changes and corrected p- values are shown in **table 1**.

In the next part of analysis, we focused on the z-scores, which tell us whether the molecular function is more likely to be decreased (negative value) or increased (positive value). The z-scores were presented as segments of inner circles in the **figure 2**. As can be seen from the figure, expression of most genes was increased (green dots) in all ontological groups. The z-scores of above-mentioned GO BP terms had positive values, so the processes described by these GO BP terms were upregulated. The expression pattern did not change at any of the analyzed time points. Considering the above, the subsequent analysis was based only on 7D/1D comparison.

FIGURE 3 The dendrogram of differentially expressed genes involved in “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility” GO BP terms. The DEGs were clustered based on their logFC values

In the next section, we checked the interaction between selected ontological groups. One of the most visually appealing way of presenting such interaction is dendrogram (**Fig. 3**). Clusters contain functionally related genes based on their expression pattern. The middle circle represents a logarithm of fold change (logFC) of differentially expressed genes assigned to the studied GO terms. The GO terms are shown as the outer ring. The genes whose expression is downregulated form clusters marked by blue part of the middle circle and analogously, red indicates upregulated genes. Clusters of the same color over the entire width of the outer circle represent genes that are unique for a specific GO term. Clusters of different colors on the cross section of outer circle show sets of genes which are likely to be functionally related. The dendrogram showed that many genes belong simultaneously to “cell motility” and “cell migration” or to “cell morphogenesis” and “cell morphogenesis involved in differentiation”. The genes that are unique for a specific GO term belong mainly to “cell morphogenesis”.

In the gene ontology database, single genes may belong to many ontological terms. For this reason, we used plots with visualization of logFC values and relationship between genes and selected GO BP terms (**Fig. 4**). The relationship was also presented as a heatmap (**Fig. 5**). The strongest upregulated genes from examined GO BP terms included, among others: *ANXA3*- annexin A3, *KIAA1199*, *HTR2B*- 5-hydroxytryptamine (serotonin) receptor 2B, G protein-coupled, *VCAM1*- vascular cell adhesion molecule 1 and *DKK1*- dickkopf WNT signaling pathway inhibitor 1.

In the next part of analysis, we focused on the interaction between proteins encoded by DEGs belonging to studied GO BP terms. Firstly, we used STRING software for the interaction prediction. The number of genes used to create STRING interaction network was limited to 50 most changed DEGs for readability (**Fig. 6**).

Finally, we used ReactomeFIViz app for investigation of functional interactions between proteins en-

FIGURE 4 Analysis of enriched gene ontological groups involved in cellular morphogenesis, junction and migration. The network plot presenting the linkages of genes and GO BP terms

FIGURE 5 Heatmap presenting the relationship between genes and selected GO BP terms. The yellow color of tiles indicates the absence of logFC values

coded by DEGs belonging to selected GO BP terms. Among the most significantly enriched functional interaction networks were FI networks for “Cell migration” and “Positive regulation of cell migration” (Figs 7 and 8).

Discussion

It is known that CCs are necessary in the process of acquiring developmental competence by the oocyte, including enabling the resumption of meiosis and the transition to the meiosis metaphase II

FIGURE 6 Interaction network of proteins encoded by 50 most changed DEGs belonging to “cell junction organization”, “cell migration”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” and “cell motility” GO BP terms. The network was generated by STRING software. Network nodes represent proteins. Empty nodes indicate proteins of unknown 3D structure

[25]. Thanks to the gap connections, it is possible to transport molecules between CCs cells and the oocyte [26]. Proper oocyte maturation without CCs is practically impossible, and the effectiveness of fertilization of such an oocyte and obtaining a correct blastocyst drastically decreases [27]. Furthermore, metabolomic studies of the spent culture medium obtained show that CCs are secreted into the external environment and allow oocyte maturation [28,29]. Due to the closeness and interaction of CCs and oocyte, they have become an interesting research model in embryology.

In the presented studies, during the transcriptome analysis of CCs cells maintained in long-term primary *in vitro* culture, groups of genes were selected, responsible primarily for processes associated with “cell junction organization”, “cell morphogenesis involved in differentiation”, “cell morphogenesis”, “cell motility”, “cell migration”. For the purposes of the presented research, these selected ontological groups can be conventionally divided into two groups: 1) “cell motility”, “cell migration” - here, genes are primarily responsible for the movement of cells from

one place to another; 2) “cell junction organization”, “cell morphogenesis involved in differentiation”, “cell morphogenesis” are responsible for organizing the components of connections between two cells but also between the cell and the extracellular matrix. In addition, a second group of selected ontological groups describes the genes responsible for cell movement from one site to the destination directed by molecular information. Moreover, genes involved in shaping cells and changing their form, shape and size that occur when non-specialized cells acquire the specialized structural features of a cell population characteristic of an organ.

The presented research results indicate that the strongest upregulated genes from examined GO BP terms included, among others: *DKK1*, *ANXA3*, *KIAA1199*, *VCAM1*, *HTR2B*. Only 6 genes demonstrated downregulation relative to the reference value, which was the gene expression shown on day 1 of primary culture *in vitro*.

The factor conditioning correct embryo implantation is activation of canonical WNT signaling. The

FIGURE 7 Reactome FI network for “Cell migration”. “--->” indicates activating/catalyzing, “-” FIs extracted from complexes or inputs and “---” predicted FIs

FIGURE 8 Reactome FI network for “Positive regulation of cell migration”. “--->” indicates activating/catalyzing, “-” FIs extracted from complexes or inputs and “---” predicted FIs

WNT signaling pathway is regulated by steroids [30,31]. WNT activation in the embryo may or may not affect the correct implantation of the embryo, but is responsible for regulating pluripotency [32]. *DDK1* is an antagonist of the WNT signaling pathway [33], as well as the protein produced by endometrial cells during the menstrual cycle, early

pregnancy or estrus in other mammals [34,35]. It is believed, that the proper development of the embryo and the proper implantation depend on a number of regulatory factors secreted by the mother’s reproductive system [36]. *DDK1* is also potentially involved in mother-embryo communication [37]. Studies on bovine embryos indicate that *DDK1* facil-

itates TE and hypoblast differentiation. *DKK1* plays one of the key roles in the proper functioning of the reproductive system of most mammals, including humans [37–41]. In the light of our research and existing knowledge about the role of CCs in communication with the oocyte, high *DKK1* expression may also affect the quality of obtained blastocyst and its implantability [42]. Studies suggest that adding CCs cells to the embryo environment improves embryo quality and pregnancy rates [43]. Perhaps the high expression of this gene in CCs cells may also affect the acquisition of oocyte developmental competences and then having sufficiently high pluripotent properties. Therefore, our research suggests that the presence of CCs cells in the oocyte environment during fertilization may result in improved *in vitro* fertilization efficiency.

The genes also responsible for the proper development of blastocyst include the *ANXA3* gene. The protein encoded by this gene is responsible for binding and maintaining primarily cell membranes. ANX are proteins found in a variety of tissues, from the respiratory and urinary epithelium to the presence in peripheral nerves. Proteins from the annexin family due to their expression in individual tissues most likely take part in the processes of equalization of individual tissues, and are also partly responsible for their physiological properties [44]. The presented studies suggest an increase in *ANXA3* gene expression in the following days of primary culture *in vitro* in the absence of oocyte. To date, the presence of the *ANXA3* gene has been reported in the morula and blastocyst stages during preimplantation development [45]. The *ANXA3* protein is part of human neutrophils and promotes tight connections between membranes of individual neutrophils, which causes their aggregation [46–48].

As mentioned in the introduction, hyaluronan (HA), or hyaluronic acid, plays an important role in the reproductive system. It is produced primarily by the oocyte, embryo and other elements of the reproductive system, depending on the species of the mammal (fallopian tube, uterus, cervix) [49,50]. In addition, HA is produced by CCs and GCs cells [51,52]. The *KIAA1199* (*CEMIP*; *HYBID*) gene showed a significant change in expression. The protein encoded by this gene plays a key role in depolymerization of HA molecules. Microarray analysis by Abe et al. identified the expression of this gene in the cochlea, as well as in the inner ear [53]. Other studies claim that this gene is responsible for promoting the process of apoptosis, cancer progression. In addition, he is responsible for regulating cell proliferation, adhesion, motility and epithelial-mesenchymal transition cells of cancer. [54].

The gene closely related to cell adhesion and extracellular matrix modeling is *VCAM1*. It shows increased expression in GCs and CCs cells [55]. High expansion of the *VCAM1* gene has also been demon-

strated in mouse granular cells after dehydroepiandrosterone (DHEA) androgenization. The result of the increase in *VCAM1* expression was an exacerbation of the symptoms of polycystic ovarian syndrome in mice (PCOS). It is suggested that *VCAM1* expression knockout is one therapeutic option for PCOS [56]. Expression of the *VCAM1* protein was not detected in mouse embryos prior to implantation. However, it is known that *VCAM1* is present in oocytes and early human embryos [57,58]. The presented research results suggest that CCs cells in *in vitro* culture medium show significant increase in expression in the following days of the *in vitro* primary culture. Perhaps the lack of interaction with the oocyte determines this growth.

Only 6 genes showed a decrease in expression relative to the control. In the presented article we focused on two genes the most downregulated (*TGFBR3* and *HMGB1*).

In the presented studies, it seems surprising that the expression of *TGFBR3* gene shows a decrease. This gene codes for the receptor protein for TGF-beta. The transforming growth factor family (TGF-beta) are multifunctional molecules involved in a number of processes primarily associated with tissue development, cell differentiation process, cell migration and adhesion, as well as the production of extracellular matrix. Receptor types I, II and III are involved in the action of TGF-beta. *TGFBR3* is a TGF-beta binding glycoprotein that can have a membrane-bound form as well as a dissolved form [59]. This receptor may also exist as an inhibin co-receptor [60]. *TGFBR3* expression in CCs cells during long-term culture decreases. This may be associated with a lack of communication with the oocyte and a change in the natural environment for CCs cells. The expression of *TGFBR3* decreases during the development of breast cancer [61]. In studies on the effect of age on the quality of oocytes, *TGFBR3* was found to be overexpressed in a group of women over 37 years of age [2]. It turns out that changing the environment to extracorporeal can have a great impact on obtaining oocyte developmental competences, as well as the development of the embryo and its proper implantation after transfer to the mother's uterus [62]. This factor can have a significant impact on the success and effectiveness of the *in vitro* fertilization procedure. The decrease in *TGFBR3* expression is associated with reduced oocyte developmental competence [63]. On the other hand, studies in mice prove that the *Tgfb3* knockout was associated with their high fertility and increased folliculogenesis [64].

HMGB1 is another gene that showed a decrease in expression. Melatonin has shown increased expression of this gene during oocyte maturation [65]. High expression of *HMGB1* reduces blastocyst demand and thus increases the chances of embryo survival [66,67]. Moreover, studies show that there

is a correlation with the amount of HMGB1 protein in follicular fluid (FF) and pregnancy and endometrial thickness. There are scientific reports confirming the fact that the protein encoded by the present gene can be an important indicator of the success of *in vitro* fertilization [68]. The protein encoded by the *HMGB1* gene is responsible for DNA binding and participates in transcription, DNA replication and repair. The decrease in expression of the presented gene may be the result of a change in the CCs cell culture environment. For transfer to *in vitro* conditions, we may suggest that these cells have lost their physiological properties [69].

Conclusions

The presented research results allowed to identify groups of genes responsible for processes related to “cell junction organization”, “cell morphogenesis involved in differentiation”, “cell morphogenesis”, “cell motility”, and “cell migration”, as well as to define interactions between individual genes. The results suggest that the most upregulated genes are primarily responsible for processes related to communication with the oocyte as well as the proper development of the embryo and its implantation. The obtained results indicate that in *in vitro* cultured CCs do not lose their properties or, on the contrary, their gene expression decreases due to the loss of contact with the oocyte.

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Conflict of interest statement

The authors declare they have no conflict of interest

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