Reproductive Sciences (2022) 29:395–409
https://doi.org/10.1007/s43032-021-00748-3

REPRODUCTIVE BIOLOGY: ORIGINAL ARTICLE

Accompaniment of Time-Lapse Parameters and Cumulus Cell RNA-Sequencing in Embryo Evaluation

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Received: 5 March 2021 / Accepted: 18 September 2021 / Published online: 12 October 2021

Abstract

The aim of this study was to investigate the use of time-lapse morphokinetic parameters and cumulus cells transcriptomic profile to achieve a more accurate and non-invasive method in embryo evaluation. Two hundred embryos from 20 couples were evaluated based on morphokinetic characteristics using time-lapse. Embryos were divided into the high-quality, moderate-quality, and bad-quality groups. Non-fertilized oocytes were considered as the fourth group. T5 (time to five cells), S2 (time from three to four cells), and CC2 (time from two to three cells) were recorded. Also, the cumulus cells of the respective oocytes were divided into high-quality, moderate-quality, bad-quality, and non-fertilized groups based on the grading of the embryos. Then their transcriptomic profiles were analyzed by RNA-sequencing. Finally, the correlation between differentially expressed genes and embryo time-lapse parameters was investigated. T5 was the only timing that showed a statistically significant difference between high-quality group and other groups. RNA-sequencing results showed that 37 genes were downregulated and 106 genes were upregulated in moderate, bad-quality, and non-fertilized groups compared to high-quality group (q value < 0.05). These genes were involved in the main biological processes such as cell cycle, DNA repair, cell signaling and communication, transcription, and cell metabolism. Embryos graded in different groups showed different transcriptomic profiles in the related cumulus cells. Therefore, it seems that embryo selection using the combination of cytokinetics and cumulus cells gene expression can improve the accuracy of the embryo selection and pregnancy rate in ART clinics.

Keywords Time-lapse · Cumulus cell · RNA-sequencing · Oocyte quality

Introduction

Is there a non-invasive but accurate method to assess the embryo quality in assisted reproductive clinics? It seems that the oocyte and her companion cells are the most probable answer to this question.

Studies have shown that the oocyte is largely responsible for regulating the fertilization process by provision of maternal proteins [1], regulation of biochemical and molecular signaling pathways [2], and transmission of nuclear and mitochondrial genome to the embryo [1]. Therefore, the oocyte quality affects embryo developmental competence to birth [3]. Since practically it is not possible to evaluate oocyte, cumulus cells (CCs) have been considered, as a powerful tool for the non-invasive assessment of developmental competency of the oocyte [4]. Studies have also shown that key molecules and signaling pathways in CCs accounts

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for embryo quality [5–7] and pregnancy outcome in human [6–8].

CCs transmit signals, as well as nutrients to the oocyte through gap junctions [9, 10]. CCs metabolize substrates such as glucose, amino acids, and cholesterol, which are essential for oocyte function [7]. The bidirectional exchanges of regulatory molecules and nutrients between CCs and oocytes are vital for the acquisition of oocyte competence and early embryonic development [11].

High-throughput RNA-sequencing (RNA-seq) has emerged as a potent tool for transcriptome analysis [12]. Due to its high sensitivity, RNA-seq has improved the quantitative and qualitative study of cell RNA content [13, 14]. RNA-seq is not limited to previous transcriptomic findings [15]. Therefore, the detection of new transcripts is facilitated [16]. In this research, RNA-seq was used to evaluate differentially expressed genes (DEG) in CCs related to high-quality, moderate and bad-quality embryos, and non-fertilized.

Time-lapse technology is a non-invasive method for assessing embryo development without inducing stress to the embryo and evaluation of possible abnormality and irregularity of developing embryos. Therefore, time-lapse has been considered a suitable tool for the de-selection of embryos with low developmental competency [17, 18].

Growing evidences have shown that CCs gene expression analysis along with time-lapse monitoring may improve our ability to assess embryo quality and offer a combined quantitative and non-invasive strategy for embryo selection. Therefore, we are aiming to evaluate the relationship between these two variables [5]. To achieve this goal, embryos were graded based on the time-lapse parameters, and embryos were divided into 4 groups including high-quality, moderate, bad-quality embryos, and non-fertilized oocytes. Finally, the transcriptomic profiles of CCs from the related oocytes were analyzed by RNA-seq, and the correlations between significantly differentially expressed genes with embryo time-lapse parameters were studied.

Materials and Methods

Patient Selection and Stimulation Protocol

In order to reduce male and female variables, as confounding factors, 20 oocyte donors with proven fertility (having at least one live birth following natural pregnancy) were included in this study. According to the rules of the clinic, women are approved as oocyte donors who do not have any diseases or abnormalities in their reproductive system and also their age and weight are in the normal range and they are not smokers. The inclusion criteria for this study were that all the donors had to have a mean age of less than 35 (31.95 ± 2.06) and $18 < \text{BMI} < 28$. The partner of the couples receiving the donated oocyte had a mean age of less than 45 (40.20 ± 3.05) DNA fragmentation index (DFI) < 19.2 [19 and with normal semen analysis according to the World Health Organization (WHO 2010) [19]. The standard antagonist-based protocol was used for ovarian stimulation. An average of 10.75 ± 3.41 oocytes was retrieved from each donor (ranging from 5 to 16).

Isolation of Cumulus Cell and ICSI Procedure

Cumulus oocyte complexes (COCs) were retrieved 36 to 38 h after the administration of hCG by ultrasound-guided the transvaginal puncture. COC was washed in GMOPS (Vitrolife, Gothenburg, Sweden) to remove blood contaminants. Then the COCs were incubated at 37 °C, in 95% humidity with 6% CO2 and 5% O2. In order to dissociate the CCs, 2 to 3 h after incubation, CCs of each COC were mechanically dissected and the corresponding MII oocytes were transferred to pre-defined labeled droplets in ICSI dish and the dissected CCs were individually transferred into a labeled cryogenic tube, centrifuged at 200 g for 5 min at room temperature and washed with phosphate-buffered saline (PBS) (Sigma-Aldrich). The remaining pellet was snap-frozen in liquid nitrogen and stored at − 80 °C until RNA extraction [20]. In this study, only CCs related to mature metaphase II (MII) oocytes were used. In order to reduce the confounding effect of oocyte immature, donor cases with higher than 10% immature oocytes were excluded from the study.

Subsequently, the MII oocytes were inseminated through conventional ICSI procedure and the inseminated oocytes were subsequently transferred to GTL medium in a defined well of Primo Vision Embryo Culture Dish under oil at 37 °C, 6% CO2, 5% O2, and 95% humidity. All the media were obtained from Vitrolife (Vitrolife, Gothenburg, Sweden). The center that this study is carried out is a referral center and gynecologist or infertility specialist makes the final decision regarding the IVF and ICSI procedure and there is more tendency for ICSI procedure and this is the reason for ICSI used in this study.

Evaluation of Sperm Apoptosis by TUNEL Assay

In order to exclude paternal factors on the embryo quality, sperms with abnormal parameters and high DFI were excluded from the study. sperm DNA fragmentation of the partners was assessed by standard TUNEL procedure (terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick end labeling, In situ Cell Death Detection Kit, Fluorescein, Roche, Germany) [21], and samples with normal TUNEL values less 19.2% [22] were included in this study. Briefly, a semen aliquot containing $3–5 \times 10^6$/mL sperm was washed with phosphate-buffered saline (PBS) and fixed by 3.7% paraformaldehyde (Sigma Aldrich).

Then samples were washed with PBS and subsequently treated with 0.2% Triton X-100 (Merck, Germany) for 5 min before being washed with an equilibration buffer. Next, according to the manufacturer's instructions, 50 µL TUNEL mix (dUTP-FITC and TDT enzyme) was used for 1 h at 37 °C in the dark. The negative control (omitting the TdT enzyme) and the positive control (incubating one sample with DNAse) were also included. Before analysis, samples were washed with PBS. For the analysis of percentage TUNEL positivity, Becton–Dickinson FACScan flow cytometer (excitation, 488 nm) was used [21].

Time-Lapse Imaging System and Embryo Quality

For this study, 200 oocytes from 20 couples were injected. 51 oocytes were not fertilized and 149 were fertilized. Therefore 149 embryos were evaluated via TLM. Image acquisition in the Primo Vision was taken every 10 min and embryos were graded according to morphokinetic parameters. According to the previous studies [23, 24], three time-lapse parameters including T5 (time to five cells (48–57 h)), S2 (time from three to four cells (< 0.75 h)), and CC2 (time from two to three cells (9–12 h)) have been identified as the most important morphokinetic parameters. According to the hierarchical algorithm, embryos are graded into 8 categories (from A^+ to D^-) [23, 24]. In group A^+, the three mentioned times are within the normal range, in group D^- these three parameters are out of the normal range, and in groups A^- to D^+, one or two of the three parameters are with the normal range. Meanwhile, embryos with deviant behaviors such as multi-nucleation in the 4-cell stage, uneven blastomere and sudden cell division into 3 or more cells, were not be included in this grading [23, 24]. In this study, we used T5, S2, and CC2 parameters according to previously defined hierarchical algorithm. The embryos with A^+, A^- to D^+, and (D^-) qualities were categorized as high, moderate, and bad-quality embryos. Unfertilized oocytes were also considered as the fourth group. Since the embryo transfers were carried out at the blastocyst stage, the morphological characteristics of the blastocysts (the quality and number of cells of both the trophectoderm (TE) and inner cell mass (ICM) and the degree of blastocoel cavity expansion) [25] were also assessed at the end of the culture period based on Gardner and Schoolcraft criteria [25], they were classified into three groups: the high-quality group with grade A, the moderate-quality group with grade B, and bad-quality group with grade C [18], and compared with time-lapse morphokinetic grading. With the exception of very few embryos, the grading was highly correlated (data are not shown), and therefore, the grading based on morphokinetic was considered for the remaining of the study.

RNA-seq and Bioinformatics Analysis

One hundred twenty CC (cumulus cell) samples collected from 12 women were selected for RNA-seq and divided into 4 groups according to embryo quality level: 33 CC samples from oocytes yielding high-quality embryos and 87 CC samples from three other groups (28, 29, and 30 samples for moderate, bad, and non-fertilized group, respectively) and finally the cumulus cells of each group were pooled together. We used the Smart-seq2 protocol for RNA-seq testing. The cells were washed twice with 1X PBS before placing in the lysis buffer. RNA was isolated from cells and converted to cDNA. Library construction was carried out following the Smart-seq2 protocol and sequenced reads that contained polyA tails, low-quality regions, and adapters were prefiltered before mapping. According to the manufacturer's instructions, cDNA was sheared into 100- to 150-bp short fragments. Libraries were pooled and sequenced on Illumina HiSeq2500 sequencers. Data normalization was performed by transforming mapped transcript reads to fragments per kilobase of transcript per million mapped reads. Genes with fragments per kilobase of transcript per million mapped reads > 0.5 were maintained for analysis [26].

Differentially expressed genes (DEGs) from each group were identified via primary analysis of RNASeq data. Significant DEGs ($p\_value < 0.05$ and $|\log2(FC)| < 1$) were used to draw volcano plots via GraphPad Prism. The analysis of 617,191 genes from RNA-seq technique was performed using the most reputable and applicable bioinformatics software and databases such as Enrichr, KEGG, UniProt, TRUFA, HGNC, and Ensembl. By comparing the moderate, bad, and non-fertilized groups with the control group (high-quality group), the genes with q value less than 0.05 (fold change of greater than 1.5) were bioinformatically evaluated. Considering the decrease and increase of genes in the transcriptome of CCs, various biological processes were investigated and the results were confirmed using several web servers and bioinformatics databases.

Expression Detection by qRT-PCR

To verify the RNA-seq results, 80 CC remaining samples collected from 8 women, that their CCs were not used for RNA-Seq analysis, were divided into 4 groups according to embryo quality: 20 CC samples from oocytes yielding high-quality embryos and 60 CC samples from three other groups (20, and 19 moderate and bad quality groups and 21 from the non-fertilized group). At first, common differential expression genes were determined. Then, three candidate genes were selected from three important pathways POLE (DNA polymerase epsilon catalytic subunit A), HAUS4 (HAUS augmin-like complex subunit 4), and AJUBA (LIM domain-containing protein ajuba). Three genes were associated with

cell cycle pathways, DNA repair, and metabolism. The RNeasy Mini kit (Qiagen, Germany) was used and total RNA was extracted from the sample. Triplicate technical replicates were carried out for all samples. All samples were treated with DNase I to eliminate contamination of genomic DNA. In order to determine the concentration and purity of RNA, the samples were analyzed using the A260/A280 ratio by a NanoDrop spectrophotometer. Reverse transcription was performed according to the manufacturer's instructions and using the High Capacity cDNA Reverse Transcription Kit (Bio-Rad, 1725037) Random Hexamer primers. The primers used in our study are presented in Table 3. qPCR reactions were carried out in triplicates using an ABI Prism 7300 Sequence Detector (Applied Biosystems, Foster City, California, USA) in a final volume of 20 µl comprising 250 ng cDNA, 5 pmol gene-specific primers, and SYBR Green reagent (Applied Biosystems). ROX dye was used as a passive control for signal intensity. The thermal cycle profile followed 50 cycles at 95° for 30 s, 60° for 30 s, and 72° for 30 s. Melting curves of PCR reactions were controlled to ensure the existence of one single PCR product without primer dimer. The efficiency of the primers was evaluated using standard curves. The SYBR Green master mix and Sequence Detection System was used to determine the relative transcript levels (Life Science, 7900HT). Gene expression level was normalized to the mouse GAPDH housekeeping gene. The ΔΔCt method was used to compute the relative quantification of gene expression and the fold change was computed as FC = 2-ΔΔCT. Prism 8 software was used for analysis [27].

Statistical Analysis

Statistical analyses were performed by one-way ANOVA and Tukey's multiple comparisons test as post hoc. Also, the SPSS version 24.0 (IBM) was used. $p < 0.05$ was considered significant.

Results

Assessing Embryo Quality on Day 5 Based on Time-Lapse Parameters

A total of 149 embryos were assessed. After morphokinetic and morphological assessment, the embryos were divided into four groups based on (Fig. 1) T5, S2, and CC2 parameters, and therefore, as described in the "Materials and Methods" section, blastocysts were categorized to high, moderate, and bad qualities. Then, moderate and bad-quality groups were compared with high-quality group. Thus, after recording the time of 3 morphokinetic parameters for each embryo in each group, the mean of the parameters was taken using SPSS software and each parameter was compared with the

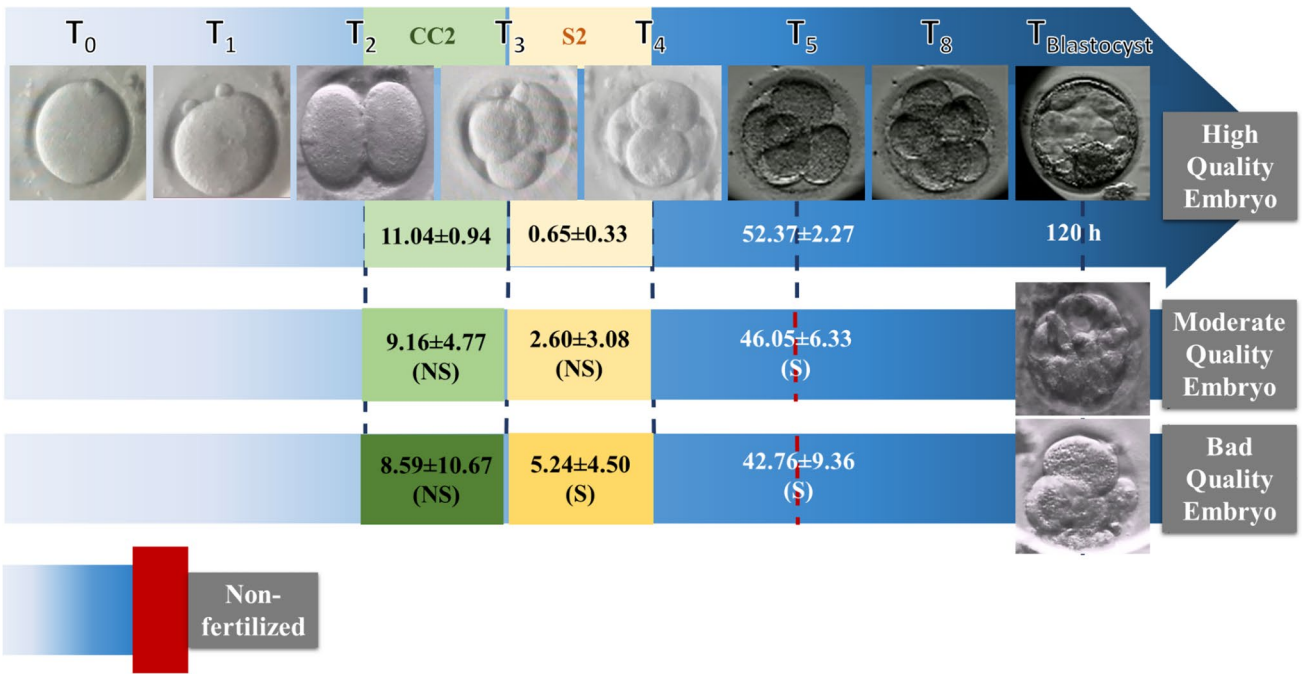

Fig. 1 Present the mean ± SD for CC2, S2 and t5 parameters in high quality, moderate and bad quality embryos. A total of 149 embryos were assessed from 20 patients and moderate (n=48) and bad quality embryos (n=48) were compared with high quality embryos (n=53). CC2: time from two to three cells, S2: time from three to four cells, T5: time to five cells. Data were shown as mean ± SD. $p < 0.05$ is considered as significant. S: significance, NS: non-significance

control group. T5 was the only timing that showed a statistically significant difference between the moderate and high-quality groups. In the bad-quality group, both T5 and S2 times were significantly different compared with high-quality group $p < 0.05$ is considered as significant (Fig. 1).

Bioinformatics Analysis

Sequencing read length was 150bp PE. After sequencing, adapter sequences (standard TruSeq LT adapter sequence) were removed and short reads trimmed. Short read sequences were then aligned to the human genome (GRCh38.87) using HISAT2. After spliced alignment, the set of expressed transcripts and differentially expressed genes was identified using Stringti and Cuffdiff. The initially analyses based on p-value identified 493, 583, and 655 significant DEGs from Moderate-, Bad-quality, and Non-fertilized vs High-quality group respectively. Figure 2 demonstrates the volcano plots of DEGs from the three groups against High-quality group. The plots indicate that there are more down-expressed DEGs in all three groups vs High-quality group. 271, 363, and 377 down-regulated DEGs are identified in Moderate-, Bad-quality, and Non-fertilized vs High-quality group respectively.

RNA-sequencing results showed that 37 genes were downregulated and 106 genes were upregulated in moderate, bad-quality, and non-fertilized groups compared to high-quality group (q value < 0.05). These differentially expressed genes were involved in the main biological processes such as cell cycle, DNA repair, cell signaling and communication, transcription, translation, and cell metabolism. A list of all the biological processes associated with these genes is presented in Table 1. Also in Table 2, was shown relative expression of up- and downregulated genes in moderate-quality, bad-quality, and non-fertilized groups versus high-quality group.

Also, GO and KEGG analyses were applied for the functional annotation and pathway analysis, using the Database for Annotation Visualization and Integrated Discovery (DAVID; https://david.ncifcrf.gov/) [28]. The human genome was selected as the background parameter. All data was shown in Fig. 3 and 4 for differentially expressed genes (DEGs) separately. To further explore the systematic characterization and biological functions of the identified DEGs, functional annotation and pathway analysis, including GO and KEGG, were performed using DAVID. The three GO categories [cellular component (CC), biological process (BP), and molecular function (MF)] were detected, respectively, using DAVID. In figure 6 also was shown schematic mechanisms of interaction between cumulus cells and oocyte in four groups (High quality, Moderate quality, Bad quality, and Non-fertilized). These 5 processes are among the most important cellular processes in which a large number of genes have been altered and their disruption may have led to changes in the quality of embryos. The red stars illustrate the intention of disruptions in the biological process.

Confirmation of RNA-seq Results by RT-qPCR Analysis

Cumulus cells related 80 oocytes from 8 patients were used for real-time PCR. As shown in Fig. 5, gene expression reflected the mRNA level for these three genes. The RT-qPCR results showed that the expression of POLE was significantly higher ($p < 0.05$) in high-quality group than other groups. However, the mRNA expression of HAUS4 and AJUBA was considerably lower in the high-quality group than other groups $P < 0.05$ is considered as significant (Table 3).

Discussion

In the present study, CCs transcriptome profile of MII oocytes was analyzed. Subsequently, based on morphokinetic characteristics of the resulting embryos were assessed using a time-lapse system. T5 was the only timing that

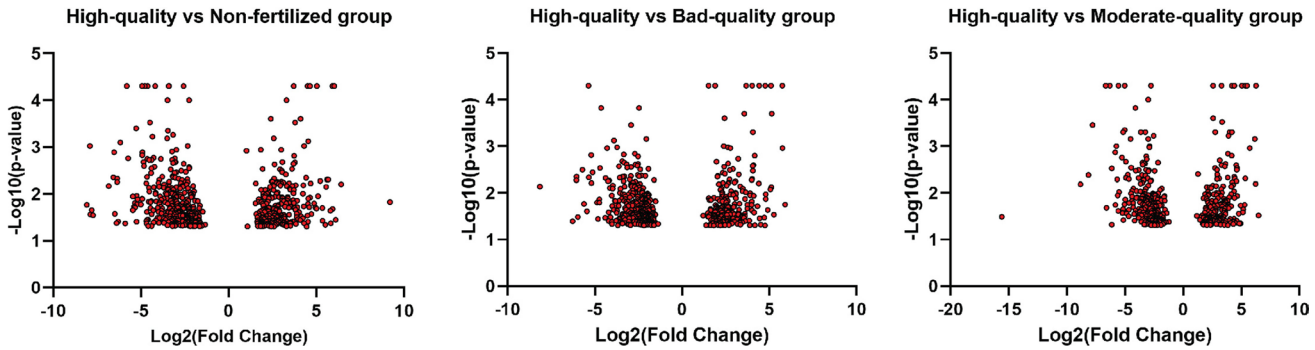

Fig. 2 The volcano plots of Non-fertilized, Bad-, and Moderate-quality vs High-quality group. The red dots show significant DEGs. Log2(FC) > 1 presents up-regulated DEGs, while Log2(FC) < -1 presents down-regulated ones. DEG, Differentially Expressed Gene; FC, Fold Change

Table 1 120 cumulus cells of 12 patients in 4 groups (High, Moderate, Bad and Non-fertilized groups) were used for RNA-sequencing. The High quality group was considered as the control group and the remaining groups were compared with it. The down- and up-regulated genes are results of the comparison of moderate, bad-quality, and non-fertilized groups with the high quality group, the genes were selected based on q-value <0.05. The biological process for each gene is also given

Biological process	Downregulated genes	Upregulated genes
Apoptosis		AEN, ITM2C, TM7SF3, TNFRSF6B, APH1A, MX1
Autophagy	ATG16L2	TECPR1
Cell adhesion	ATXN3, CEP162	ARHGDIB, VASP, ACAN, AJUBA, ADAM15, NINJ1
Cell cycle	FBXL8, PKMYT1, LLGL2, POLE	AJUBA, HAUS4, AKAP8L, L3MBTL2, RTEL1, RTEL1-TNFRSF6B, KLHDC8B
Cell signaling and communication	MAP4K2, DAB1, SNTG2, ADGRV1	AJUBA, RASA4B, RASA4, STK19, MUC20, MUC20-OT1, GABBR2, CACNB3, PKD1, ERBB2, FGFR1, LTBP4, TTC36, RGS12, NPIPB3, NPIPB4, RASGRP2, UPK3BL1, TRIP10, KALRN, IQSEC2, EPN2, COL2A1
Disease perturbation (polycystic ovary syndrome)	–	SNHG1, SNORD22, SNORD25, SNORD26, SNORD27, SNORD28, SNORD30, SMG1P3, NPIPB3, U2AF1L4, TLE4
DNA repair	MUTYH, SMUG1, POLE, NEIL1, ATXN3	RTEL1, RTEL1-TNFRSF6B, GTF2H2, AEN, ENDOV
Histone modifications	BRCA1	
Inflammatory response		C4A
Ion and protein transport	MYO1C, MYO15B, STIM1, SLC37A3	CORO7, CORO7-PAM16, PAM16, EVI5L, CORO7, ACD, PITPNM1, RPH3AL
Metabolic process	OMA1, MCEE, ABHD14A, ABHD14A-ACY1, ACY1, PRPSAP2, ALG13, CBWD5	HEXA, NR1H3, ECHDC2, AJUBA, TM7SF3, CRYZL1, FGGY, PLA2G6, SLC52A2, PITPNM1, LRTOMT, BLVRB, PAFAH1B3, GALNT3, PEX5L, GALE
Post-translational modification	KLHL11	QARS (EPRS1), RABGGTA, LCMT2, Sep-04 (SEPSECS), DUS3L, ASNA1 (GET3), USF2, PARP6
Proteolysis	ATXN3, ALG13	DPP9, CPB1, APH1A
Transcription and translation	HSF4, ZNF559, PRPF18, ZNF177, ZNF559-ZNF177, ZNF195	POLR2J3, POLR2J2, NR1H3, AJUBA, CELF6, USB1, AKAP8L, U2AF1L4, ZNF536, RFX2, TLE4, FGFR1, NFATC1, USF2, PARP6, RABGGTA, TSEN54

showed a statistically significant difference between the moderate and high-quality groups. In the bad-quality group, both T5 and S2 times were significantly different compared with high-quality group (Fig. 1). Comparison of the transcriptional analysis revealed that a number of important genes involved in the main biological processes such as cell cycle, DNA repair, cell signaling and communication, transcription, translation, and cell metabolism were differentially expressed (Table 1). Analysis of the RNA-seq data showed that in moderate- and bad-quality groups, the trend of gene transcription was altered in the CCs of each group compared to high-quality group. Moreover, in the non-fertilized group, a more severe imbalance of the transcriptome was observed (Fig. 6). Because these cells have a pivotal role in oocyte development and fertilization, changes in genetic integrity and disruption in the main biological processes of these cells could influence oocyte quality and the resulting embryo [9]. Studies had shown a significant negative relation between sperm parameters, sperm DNA fragmentation and embryo development[30]. Therefore,In order to exclude paternal factors on the embryo quality, sperms with abnormal parameters and high DFI (> 19.2) were excluded from the study.

Correlation Between Metabolism Process in Cumulus Cells and Embryo Quality

Metabolism is a main determining factor of oocyte and preimplantation embryo viability. But the oocyte cannot undergo a series of metabolic processes of energy production, including glucose metabolism, cholesterol synthesis, and supply of alanine. The CCs provide the oocyte with pyruvate, cholesterol, and amino acids as substrates [10]. In this study, the expression of many genes involved in

Table 2 The relative expression of up- and downregulated (−) genes in moderate-quality, bad-quality, and non-fertilized groups versus high-quality group. Bar represent (1/*q* value) amount to ease the comparison of relative expressions. *q* value < 0.05 was considered as significant (*p* value < 0.05 and |log2(FC)| < 1)

Gene	Moderate		Bad		Non-fertilized	
	Fold change	*q* value	Fold change	*q* value	Fold change	*q* value
ABHD14A					−9.80671	0.028019
ABHD14A-ACY1					−9.80671	0.028019
ACD					10.50015	0.028019
ACY1					−9.80671	0.028019
ACAN	11.068	0.028019				
ADAM15					9.487145	0.028019
ADGRV1					−10.0811	0.028019
AEN	9.376859	0.028019	9.576762	0.028019	9.891368	0.028019
AJUBA	6.258637	0.028019	5.74146	0.028019	6.02871	0.028019
AKAP8L	10.58175	0.028019	10.88904	0.028019	10.88093	0.028019
ALG13					−3.48741	0.047837
APH1A					10.71691	0.028019
ARHGDIB	10.51639	0.028019				
ASNA1			3.51622	0.028019		
ATG16L2	−9.68711	0.028019				
ATXN3					−2.57748	0.028019
BLVRB					12.64486	0.028019
BRCA1	−5.55433	0.028019				
C4A	9.345769	0.028019			9.353795	0.028019
CACNB3			9.421343	0.028019		
CBWD5					−4.78105	0.028019
CELF6	4.187849	0.028019	4.76368	0.028019	4.50222	0.028019
CEP162					−9.60901	0.028019
COL2A1					10.33703	0.028019
COR7	4.293181	0.028019			4.63758	0.028019
COR7-PAM16	4.393111	0.028019			4.63600	0.028019
CPB1					9.519269	0.028019
CRYZL1			9.33024	0.047837		
DAB1					−9.60947	0.028019
DPP9					10.67298	0.028019
DUS3L	8.41188	0.028019				
ECHDC2	9.643371	0.028019	10.30569	0.028019	10.97422	0.028019
ENDOV					11.17837	0.028019
EPN2					5.9189	0.028019
EPRS1			5.32892	0.028019		
ERBB2			9.431017	0.028019		
EVI5L			9.755759	0.028019		
FBXL8	−9.40282	0.028019	−9.41381	0.028019		
FGFR1			4.01903	0.028019	3.72091	0.028019
FGGY			9.831907	0.028		
GABBR2			9.449254	0.047837		
GALE					12.21399	0.028019
GALNT3					10.42194	0.028019
GTF2H2					9.583617	0.028019
HAUS4	6.248137	0.028019	5.73246	0.028019	6.12871	0.028019
HEXA	4.187249	0.028019	4.26318	0.028019	4.48252	0.028019
HSF4	−9.42082	0.028019	−9.41581	0.028019		
IQSEC2					9.329388	0.028019
ITM2C			8.469348	0.028019	9.549358	0.028019

Table 2 (continued)

Gene	Moderate		Bad		Non-fertilized	
	Fold change	*q* value	Fold change	*q* value	Fold change	*q* value
KALRN					9.612828	0.028019
KLHDC8B					10.94287	0.028019
KLHL11			−9.35667	0.047837		
LCMT2	10.73882	0.028019				
LLGL2					−9.27785	0.047837
LRTOMT					9.680897	0.028019
LTBP4			10.04227	0.028019		
L3MBTL2			10.03993	0.028019		
MAP4K2	−10.4867	0.028019				
MCEE					−10.7863	0.028019
MUC20			1.51482	0.028019		
MUC20-OT1			1.51482	0.028019		
MUTYH	−10.3931	0.028019			−10.5921	0.028019
MX1					9.303966	0.028019
MYO1C	−5.02161	0.028019	−5.35674	0.028019	−4.61502	0.028019
MYO15B	−11.2178	0.028019				
NEIL1					−9.71432	0.028019
NFATC1					10.55464	0.028019
NINJ1					12.94752	0.028019
NPIPB3			5.0905	0.028019		
NPIPB4			10.79335	0.028019		
NR1H3	10.15086	0.028019	10.02475	0.028019	11.16325	0.028019
OMA1					−9.60947	0.028019
PAFAH1B3					11.80034	0.028019
PARP6	4.187849	0.028019	4.36368	0.028019	4.50222	0.028019
PEX5L					9.929019	0.028019
PITPNM1					11.38677	0.028019
PKD1			10.08027	0.028019		
PKMYT1			−9.9372	0.028019	−9.9372	0.047837
PLA2G6			10.15919	0.028019	9.920258	0.028019
PAM16	4.493121	0.028019			4.52889	0.028019
POLE	−9.1329	0.028019	−9.2188	0.028019	−10.3309	0.028019
POLR2J2	3.299729	0.028019			5.12773	0.028019
POLR2J3	3.192799	0.028019			5.02783	0.028019
PRPF18	−2.77773	0.028019			−2.25175	0.047837
PRPSAP2					−3.39191	0.028019
RABGGTA	10.32764	0.028019			11.49897	0.028019
RASA4	3.299799	0.028019			5.02773	0.028019
RASA4B	3.391739	0.028019			5.12573	0.028019
RASGRP2					9.5416	0.028019
RFX2			9.86594	0.028019	9.813412	0.028019
RGS12			9.351405	0.028019	9.754495	0.028019
RPH3AL					9.283014	0.028019
RTEL1					12.26301	0.047837
RTEL1-TNFRSF6B					12.26200	0.047837
SEPSECS			4.33425	0.028019		
SLC37A3					−5.80501	0.028019
SLC52A2					11.59633	0.047837
SMG1P3			5.0905	0.028019		

Table 2 (continued)

Gene	Moderate		Bad		Non-fertilized	
	Fold change	*q* value	Fold change	*q* value	Fold change	*q* value
SMUG1					− 10.827	0.028019
SNTG2					− 13.0114	0.028019
SNORD25	9.175690	0.028019			9.994067	0.028019
SNORD26	9.273301	0.028019			9.894067	0.028019
SNORD27	9.475001	0.028019			9.123230	0.028019
SNORD28	9.333701	0.028019			9.994066	0.028019
SNORD30	9.275701	0.028019			9.89406	0.028019
STK19	9.345769	0.028019			9.353795	0.028019
SNHG1					8.894067	0.028019
SNORD22					9.893322	0.028019
STIM1			− 10.1735	0.047837		
TECPR1					11.68414	0.028019
TLE4			9.987221	0.028019		
TM7SF3			3.67741	0.028019		
TNFRSF6B					12.26301	0.047837
TRIP10					10.72161	0.028019
TSEN54					10.59344	0.047837
TTC36			9.510677	0.028019		
U2AF1L4	10.08397	0.028019	9.419924	0.028019		
UPK3BL1	3.299799	0.028019			5.02773	0.028019
USB1	10.35807	0.028019			11.31755	0.028019
USF2	10.08811	0.028019			10.36385	0.028019
ZNF177	− 3.1263	0.047837			− 4.4163	0.028019
ZNF195					− 4.93701	0.028019
ZNF536			9.753949	0.047837		
ZNF559	− 3.3323	0.047837			− 3.4363	0.028019
ZNF559-ZNF177	− 3.1363	0.047837			− 3.5161	0.028019
VASP	10.73433	0.047837				

metabolic pathways, such as OMA1, NR1H3, and ACY1, was altered. The OMA1 gene, among the genes involved in oocyte maturation [28] and ACY1 (Aminoacylase 1), involved in amino acid metabolism pathway [18], had the highest expression in the high-quality group and the lowest expression in the non-fertilized group.

The NR1H3 (Nuclear Receptor Subfamily 1 Group H Member 3) gene participates in the metabolism of carbohydrates, lipids, and cholesterol and plays an important role in the pathophysiology of many metabolic diseases. This gene is involved in cholesterol metabolism by suppressing cholesterol synthesis [29].

NR1H3 was upregulated in moderate, bad, and non-fertilized groups compared to the high-quality group. Increased expression of this gene may lead to a decrease in cholesterol synthesis in cumulus cells and thus a cholesterol reduction in the corresponding oocytes. Since the embryo is not able to synthesize cholesterol until the 8-cell stage, it probably needs cholesterol stored in the oocyte for development [30]. Cholesterol deficiency in the embryo before implantation result in slow cleavage rate and prevent progressing to the blastocyst stage [31]. Therefore, the possible deficiency of cholesterol in the cumulus cells and oocytes may be one of the reasons for the S2 longer and the difference in time of cleavage divisions in moderate and bad groups compared to the high-quality group. Hammond et al. also showed that genes involved in metabolism, especially the glycolytic pathway in CCs, are correlated to time-lapse parameters which may impact on the speed of cleavage [5]. Disruption of this process in CCs leads to a decrease in oocyte competence and eventually embryo development.

Cumulus Cells Apoptosis Is Related to Moderate and Bad-Quality Embryos and Non-fertilized Oocytes

One of the altered pathways in our study was the apoptotic pathway. Our results showed that although the expression of some antiapoptotic genes have increased, the number of pro-apoptotic genes that have been altered is greater,

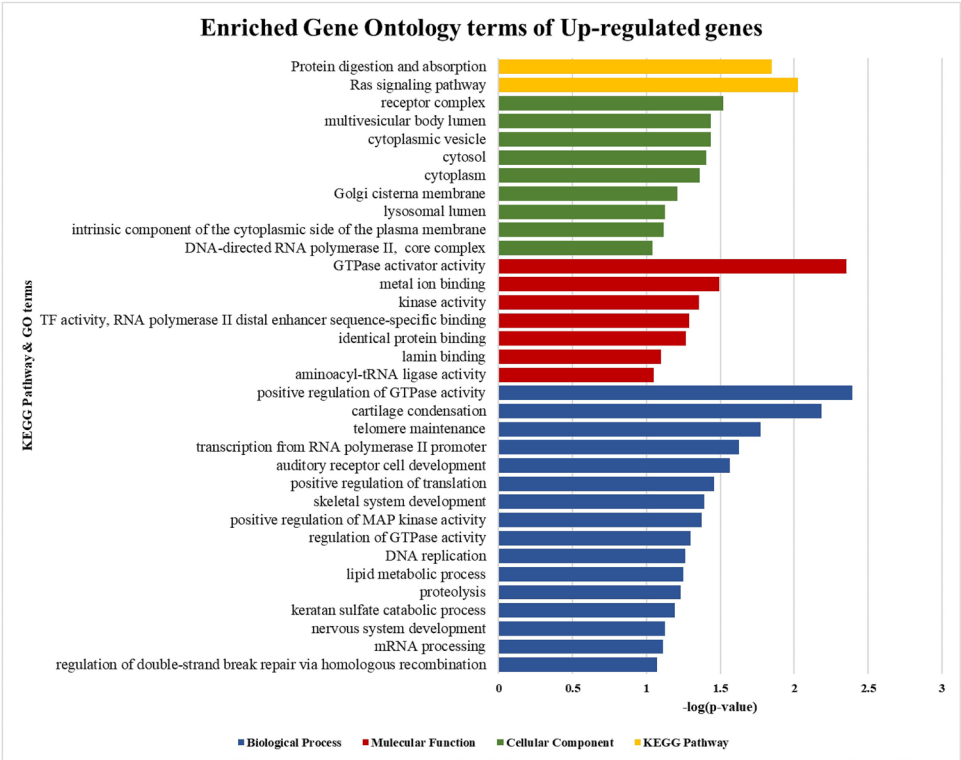

Fig. 3 Enriched Gene Ontology terms of Up-regulated genes. This presents mostly participated biological pathways and processes by Up-regulated genes. TF, transcription factor. CC, cellular component. BP, biological process. MF, molecular function. KEGG, kyoto encyclopedia of genes and genomes

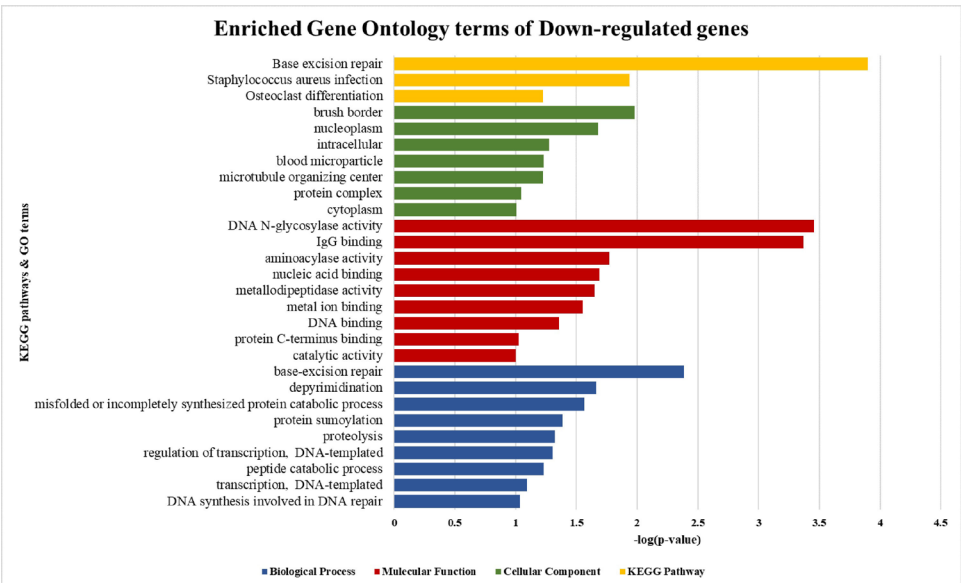

Fig. 4 Enriched Gene Ontology terms of Down-regulated genes. This presents mostly participated biological pathways and processes by Down-regulated genes. TF, transcription factor. CC, cellular component. BP, biological process. MF, molecular function. KEGG,kyoto encyclopedia of genes and genomes

and this balance may lead to an increase in apoptosis. In this study, the expression of the number of apoptosis-inducing genes including AEN (apoptosis-enhancing nuclease) and ITM2 (integral membrane protein 2) in CCs related to moderate, bad (with longer S2 and shorter CC2 and T5), and non-fertilized groups was increased. As apoptosis in cumulus cells increases, the oocyte may not receive proper sustenance from these cells [32]. Increase in CCs apoptosis has been also accompanied with morphologically abnormal oocytes [32], disrupted fertilization [33], and suboptimal blastocyst development [34]. Another study suggested the association between increased expression of apoptotic genes in CCs with abnormal cleavages and longer T2 in embryos [35]. Therefore, increased apoptosis in cumulus cells may lead to the formation of less competence oocytes and ultimately disruption of the

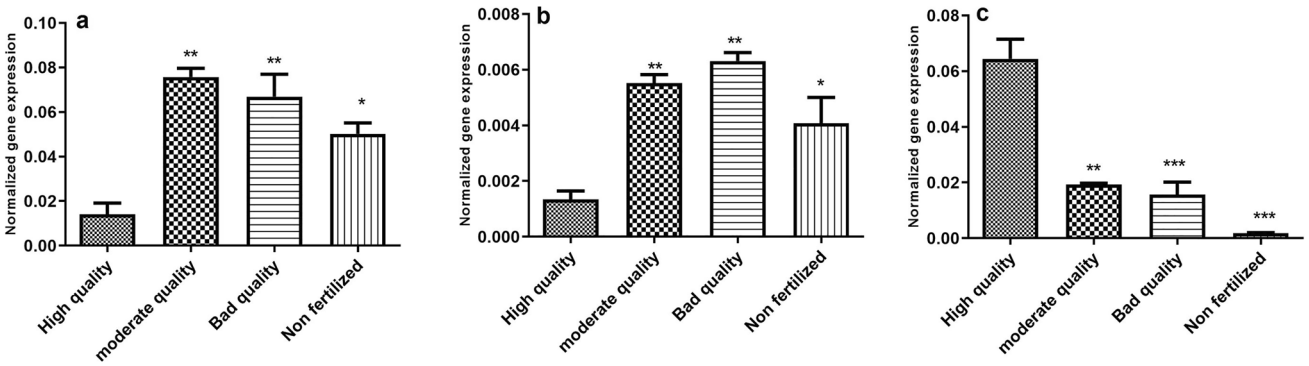

Fig. 5 This figure shows the relative expression of three candidate genes, each representing one pathway in the cumulus cell of oocytes resulting in high, moderate and bad quality embryos or failed fertilization following ICSI. The results of the latter groups (moderate(n=20), bad(n=19) and non fertilized(n=21)) groups were compared with high quality group(n=20). Cumulus cells related 80 oocytes from 8 patients were used for real-time PCR. a HAUS4, b AJUBA, c POLE. Each bar represent triplicates and *P< 0.05 is considered as significant

Table 3 Sequence of primers used in the current investigation

Gene	Forward primer (5–3)	Reverse primer (5–3)
HAUS4	AGCTTCTCCTGAATCTCTCACA	GTGTCTTCACATAGTAGTCCACA
AJUBA	TCCTCCCACCTTGTCCTCCCA	CACTACATCAACCACAACCCCA
POLE	TTGTCAAAGTGAGGAAGGAGA	AGAGGAGGATGTGGTAGGGA
GAPDH	GCA GGG ATG ATG TTC TGG	CTT TGG TAT CGT GGA AGG AC

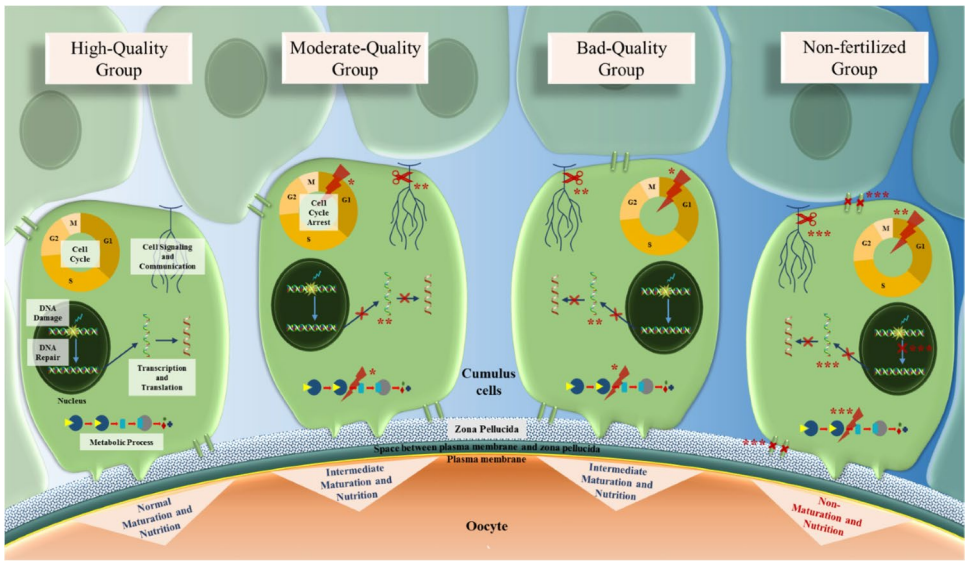

Fig. 6 Schematic mechanisms of interaction between cumulus cells and oocyte. Mechanisms of cumulus cells and oocyte interaction have been shown in four groups (High quality, Moderate quality, Bad quality, and Non-fertilized). The red stars illustrate the intention of disruptions in the biological process

cleavage divisions especially T5 and CC2 of our moderate and bad-quality embryos in this study.

Negative Effect of Altered Cellular Signaling and Communication Pathways on Embryo Quality

Bidirectional signaling between CCs and oocyte remains after ovulation and even fertilization [36]. In the current study, genes involved in signaling and cellular communication processes were altered in bad, moderate, and non-fertilized groups compared to the high-quality group. The DAB1 gene was one of these genes that was downregulated in the non-fertilized group [37]. It has also been shown that DAB1 downregulation in cumulus cells contributes to decreased nutrient uptake by the oocyte [38]. Defects in this gene may lead to defects in oocytes competence of

non-fertilized group. AJUBA, which is one of the common differential expression genes in our study, plays important roles in the cell cycle and communication pathway. The AURKA(Aurora Kinase A), which is involved in regulating the cell cycle of oocytes, is dependent on the LIM-domain protein Ajuba for regulation of its kinase activity function[42, 43]. Alteration in the expression of AJUBA in cumulus cells may affect the cell cycle of oocyte and embryo. AJUBA,

Also, MAP4K2, which is involved in the interaction between the oocyte and CCs [39], decreased in the moderate group. In moderate-quality embryos, T5 was also significantly different from the high-quality group. Numerous studies have shown a significant relationship between T5 parameter and blastocyst formation [40, 41]. Defects in the signaling pathway between the oocyte and the CCs may lead to a decrease in oocyte competence and defects in cleavage divisions, especially T5. In line with our findings, it has been reported that CCs genes involved in signaling pathways were positively correlated to time-lapse parameters [5]. This finding supports the idea that the ovarian microenvironment in which the oocyte is formed affects the developmental timings of the embryo [5].

Altered Expression of Transcription Pathway Genes in Moderate, Bad, and Non-fertilized Groups

Cumulus cells transfer RNA to the oocyte [42, 43], which is pivotal for the supply of maternal reserves [42] because until the embryo genome (EGA) is activated, embryos are dependent on oocyte RNAs for development [44]. Among the important altered genes in the current study were zinc finger family (ZNF) genes that are involved in transcriptional regulation. Investigations have shown that some of ZNF genes are expressed in oocytes and early embryonic stages and play a critical role in embryo development [44, 45]. Since embryonic quality is determined in the early stages of development and is inherited from the oocyte [5], defects in the expression of these genes in CCs may affect oocyte RNAs supply and ultimately impair embryo development.

The Effect of Changes in DNA Repair and Cell Cycle Pathways of Cumulus Cells on the Embryo Quality

Some studies have shown that the oocyte competence is correlated to the DNA status of CCs [46] and oocyte ability to reach the blastocyst stage may be impaired by DNA fragmentation in CCs [47, 48]. Among the genes altered in our study were SMUG1 (Single-strand selective monofunctional uracil DNA glycosylase) and MUTYH (mutY DNA glycosylase) genes, which are involved in DNA repair and have a higher expression in the high-quality group. Studies have shown that these two genes are also expressed in oocytes and are involved in DNA repair [49]. When DNA fails to repair double-strand break, it can lead to apoptosis and cell cycle arrest [48].

Also, POLE gene, which has been reduced in moderate, bad and non-fertilized groups compared to the high quality group, is one of the DNA polymerases involved in DNA repair, which is also involved in ovary cancer[55]. Abnormalities in cell cycle regulators can have devastating consequences. HAUS4 localizes to the spindle microtubules and has a role in mitotic spindle assembly and centrosome integrity during cell division. Changes in the expression of this gene in this study may lead to a change in the quality of oocyte and embryo[56]. The PKMYT1 (Membrane-associated tyrosine- and threonine-specific cdc2-inhibitory kinase) gene regulates the cell cycle, which in our study, were down-regulated in the bad and non-fertilized groups compared to the high-quality group. PKMYT1 promotes the cell cycle in somatic cells, and its deficiency impairs cell proliferation and increases of apoptosis rate [50]. Increased apoptosis in cumulus cells can lead to abnormalities in the cleavage divisions of embryos and may be one of the reasons for deviating from the normal range of T5 and S2 in embryos of the bad group. Research has shown that a decrease in PKMYT1 in *Xenopus*'s oocyte leads to precocious oocyte maturation. Such oocytes are fertilized but are arrested in the single-cell stage [51]. Decreased expression of this gene in the non-fertilized group may lead to their arrest. Also, PKMYT1 has an important role in controlling meiotic resumption of the mouse's and *Xenopus*'s oocyte (through meiosis-promoting factor inhibition) [52, 53]. The mechanisms controlling cell division in the oocytes may be affected by some genes identified in CCs [5]. Hence, the cell cycle in CCs may be a mirror of the cell cycle in the oocyte. Further investigation may link the cell cycle in CCs to the oocyte quality and eventually cell divisions of embryos.

Some studies have investigated the relationship between cytokinetic parameters and blastocyst formation. They showed that there is a positive relationship between primary cleavage divisions and the time intervals between divisions with the blastocyst formation potential [50, 51]. On the other hand, in some studies, a correlation between the CCs gene expression and embryo quality has been investigated [24, 52]. But the simultaneous use of two parameters has been less studied. Also, in all these studies, a limited number of genes have been examined [5, 31, 53]. In this study, for the first time, using the RNA-seq technique, this relationship has been assayed more broadly. In this study, all oocytes were MII in terms of nuclear maturity, although the CCs associated with them showed different transcriptional profiles, and the respective embryos were also grouped into different groups in terms of quality and grading. The results showed that cytokinetic parameters in the high-quality group were

significantly better than other groups. Also, the transcriptomic profile of this group was different from other groups. Therefore, it seems that embryo selection using the combination of cytokinetics and CCs gene expression assessment can improve the accuracy of the embryo selection and pregnancy rate in ART clinics. Despite the strengths of the present study, its main limitation was the pool of the cumulus cells from each group. In addition, due to the high volume of information, intra-group study data can be examined in a separate study.

Conclusion

These results suggest a new prospect to the determinants of oocyte quality and embryo selection, as well as provide new insight which may eventually be used to clarify the variables that affect early embryo development and improve embryo culture medium.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s43032-021-00748-3.

Acknowledgements The authors would like to thank Iran University of Medical Sciences (IUMS), Tehran, Iran, for their cooperation throughout the period of study.

Author Contribution MM: project administration, designed the study, and reviewed the manuscript. AG: performed the experiments and wrote the paper. FSA: designed the study and reviewed the manuscript. MHN: supervised and reviewed and editing the manuscript. ER: analyzed data.

Funding This study was financed by the co-operative of Iran University of Medical Sciences (grant no. 32819) and Iran National Science Foundation (grant no. 95849005).

Data Availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Code Availability Not applicable.

Declarations

Ethics Approval The study approval was obtained from the Ethics Committee of Iran University of Medical Science (reference number: IR.IUMS.FMD.REC.1397.148). Also, samples were collected from patients in Isfahan Fertility and Infertility Center.

Consent to Participate Informed consent was obtained from all participants included in the study.

Consent for Publication All authors consent to publication of this study.

Conflict of Interest The authors declare no competing interests.

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