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Intracytoplasmic sperm injection outcomes and gene profile alteration in human cumulus cells related to advanced maternal age

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Abstract: Cumulus cells (CCs) play an important role in oocyte mutation and development potential. They have been reported beneficial for oocyte selection, which is crucial for improving the success rate of assisted reproduction technology (ART). Here, we studied gene expression profile shifting in CCs affected by female ageing. CCs from 151 cases of intracytoplasmic sperm injection (ICSI) patients were collected, divided into younger (55 cases, ≤35 years old) and older (37 cases, 36-40 years old; 59 cases, >40 years old) groups. Based on ICSI results, the fertilization rate, embryo development rate and clinical pregnancy rate were negatively correlated with age. Using RNA sequencing, gene expression profile shifting occurred in CCs from different maternal ages. In total, 193 genes were differentially expressed between age groups. Gene ontology annotation shows that 64 genes were annotated to the developmental process and 20 genes were related to the reproductive process. We analysed differentially expressed genes related to reproduction and constructed protein interaction networks to selected crucial gene nodes. Furthermore, qRT-PCR validation was performed for four key node genes. This study provides a set of genes that might be beneficial for oocyte selection during in-vitro fertilization (IVF) and clinical outcomes.
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Keywords: Cumulus cells; Advanced maternal age; Assists reproduction technology; Transcriptome; Clinical outcomes

1. Introduction

As a common disease, the rate of infertility continues to increase and has reached approximately 8-10 % prevalence due to multiple effectors according to World Health Organization (Suneeta et al., 2012). Female infertility is affected by stress, general health, sexually transmitted diseases (STDs), tubal disease, endometriosis and advanced maternal age (AMA) (Resolve; Healy, 1994; Jackson et al., 2015; Rooney & Domar, 2018). Based on the development for more than 35 years, assisted reproductive technologies (ART) such as in-vitro fertilization (IVF) have improved the chances for pregnancy and are accepted as an efficient treatment for infertility (Bhattacharya, 2003). As one of the most significant risks for female infertility, AMA has decreased of pregnancy loss and increased spontaneous abortion in spontaneous or assisted reproductive attempts (Pantos et al., 1999; Sauer, 2015). The risk of AMA is related to the reduced quality of oocytes and embryos, and a high risk of spontaneous abortion from aneuploidy (O'Brien et al., 2017). The ageing oocyte has a risk of increasing mitochondria DNA damage, decreasing oxidative phosphorylation and ATP synthesis (McReynolds et al., 2012).

Cumulus cells (CCs) are a special cluster of somatic cells that encircle the oocytes and perform specialized roles in oocyte growth and maturation. They mediate the microenvironment of ovarian follicles, direct contact with oocytes via gap junctions and form cumulus-oocyte complexes (COCs). The communication between CCs and oocytes is critical for the maturation of oocyte nuclear and cytoplasmic that determine the competency of fertilization; communication also regulates the embryo development during early stage and fecundity. Therefore, gene expression analyses of CCs have the potential for non-

invasively identifying novel biomarkers of oocyte competence, embryo quality and pregnancy outcomes (Assou et al., 2010; Fragouli et al., 2012; Huang & Wells, 2010). Many studies have focused on the transcriptome profile shift of CCs for oocyte and embryo quality, and IVF outcomes (Iager et al., 2013; Jia et al., 2019; Liu et al., 2018; Wathlet et al., 2011; Yerushalmi et al., 2014). Although several studies have been designed to investigate the influence of maternal age on oocyte quality and developmental potential, the pregnancy outcomes were not clear in these studies (Al-Edani et al., 2014; Molinari et al., 2016).

AMA is defined as a pregnant woman >35 years old; it has been reported that this age is the demarcation of decreased fecundity and acceleration of infertility (Harrison et al., 2017; Healy, 1994; Jackson et al., 2015). We grouped patients into younger (≤35) and older (>35) to evaluate the clinical outcomes. We applied a high throughput transcriptome study to gain insight into the reproduction-related gene difference in CCs derived from maternal age. Furthermore, a systematic bioinformatics analysis was established to explore the pathways and networks of genes related to the reproduction process.

2. Material and Methods

Patients

In this study, a total of 151 infertility patients enrolled in ART treatment at Liuzhou Maternity and Child Healthcare Hospital (Liuzhou, Guangxi, China) from March 2015 to May 2017 were prospectively studied. All patients were subjected to extensive gynaecological and cytogenetic examinations; we applied strict exclusion criteria to eliminate potential bias. The exclusion criteria include polycystic ovary syndrome (PCOS), endometriosis, premature ovarian failure and any metabolic, cardiovascular, autoimmune or oncological pregestational maternal disease. The subjects were divided into two groups: younger (≤35 years, mean of 27.4) and older (36-40 years old, mean of 36.6; >40 years old, mean of 42.8) to study the outcome of ART influenced by maternal age. Table 1 shows the clinical characteristics of each age group. This study was approved by the Institutional Review Board of the hospital and was carried out in accordance with The Code of Ethics of the World Medical Association for experiments involving humans; we obtained informed consent from all patients.

Table 1. Clinical characteristics of the studied groups. (Data are the mean ± SD of each age group of patients. And CCs of each groups were pooled and divided into two biological replicates for RNA-sequencing.)

Variable	Younger women (≤ 35 years old)	Older women (> 35 years old)	
		36~40 years old	> 40 years old
Number of patients (n)	55	37	59
Age (years)	27.4 ± 1.9	36.6 ± 2.3	42.8 ± 1.6
Year of infertility	3.2 ± 0.9	3.9 ± 1.1	5.7 ± 5.6
FSH (I.U·L-1)	5.8 ± 1.2	6.3 ± 1.0	8.0 ± 4.2
LH (I.U·L-1)	4.5 ± 0.9	4.7 ± 1.3	3.7 ± 3.6
E2 (ng·L-1)	43.3 ± 5.5	45.4 ± 0.9	50.6 ± 63.9
Antral follicle counts (n)	13.8 ± 2.5	11.1±2.0	4.5 ± 3.1

ART procedures and cumulus cells isolation

For ovarian stimulation, all patients were treated with gonadotropin-releasing hormone agonist started on the fourth day of the menstrual cycle. The doses were adjusted based on ultrasound monitoring and serum oestradiol (E2) detection. When three dominant follicles reached 18 mm in diameter, a dose of 5,000–10,000 I.U. of human chorionic gonadotropin (hCG, Serono, Germany) was applied for inducing ovulation. The cumulus-oocyte complex (COC) retrieval was performed with follicular puncture under the guide of transvaginal ultrasound 36–38 h after induction. COCs collected for IVF were dealt with 80 I.U. hyaluronidase (Sigma, America) to remove cumulus cells. The cumulus cells derived from matured COCs were washed with PBS and centrifuged at 235 g for 6 min at 4 °C (Kedem et al., 2014). The resulting pellet was stored at -80 °C for RNA extraction. Cumulus-free oocytes were selected for the conventional intracytoplasmic sperm injection (ICSI) procedure. Following ICSI, successfully fertilized oocytes were selected for developing a culture in G1-PLUS™/G2-PLUS™ sequential medium (Vitro Life, Sweden) for 3 days. Then, the high-quality embryo was selected for transfer.

Outcome variables

The primary IVF outcomes of the participants include serum FSH, LH, and E2 level on the day of hCG injection, the endometrial thickness on the progestin day, dipronuclear (2PN) cleavage rate, fertilization rates and good-quality embryos. For pregnancy assessment, an hCG detection combined with ultrasound observation of gestational sacs were

applied for defining the clinical pregnancy rate. The spontaneous miscarriage rate was defined as those who suffered a pregnancy loss before 12 weeks of gestation.

Total RNA extraction and sequencing

Total RNA was extracted using Trizol reagent (Takara, Dalian, China) according to the manufacturer's instruction. Briefly, pooled CCs of the differential group were homogenised in 500 µl Trizol reagent and incubated at room temperature for 5 min. After 15 min centrifugation at 12,000 g and 4 °C, the supernatant was added with 100 µl chloroform and the sample was vortexed for 15 min. RNA was precipitated with 250 µl isopropanol and the pellet was collected under another 10 min centrifugation at 12,000 g and 4 °C. Finally, the RNA pellet was washed with 75% ethanol 3 times, dried and dissolved in 20 µl RNase-free water. The RNA concentration was measured by nanodrop and the RNA quality was assessed on Agilent Bioanalyzer 2100. The cDNA library was constructed following the NEBNext® Ultra™ Directional RNA Library Prep Kit protocol. Libraries were sequenced on an Illumina HiSeq 2500 platform and paired-end 150-bp reads were generated for the following analysis. The reads were aligned to the reference genome database downloaded from NCBI. Data normalisation was carried out by transforming the mapped transcript reads to FPKM.

Gene expression validation by quantitative RT-PCR

For cDNA template construction, 100 ng total RNA was used according to the instruction of a high capacity reverse transcription kit (Applied Biosystems). The quantitative real-time PCR reaction was performed on a qTOWER2.0 System (Analytik Jena, Germany). The real-time PCR assay was performed in a 20 µl total reaction volume containing 2 µl cDNA, 2 µl forward primers and 2 µl reverse primers (Table S1); 10 µl of 2 × SYBR Green PCR Master Mix (Applied Biosystems) was added with 4 µl RNase-free water. PCR reactions were performed in three replicates. Universal thermal cycling parameters (initial step of 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 30 s at 60 °C) were used. The expression datasets of all candidate genes were normalised to the endogenous housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the $\Delta\Delta$Ct method.

Bioinformatic analysis

Genes were classified by Gene Ontology annotation, which was composed of three categories: biological process, cellular component and molecular function. Gene Ontology (GO) annotation was derived from the UniProt-GOA database (http://www.ebi.ac.uk/GOA/). The interaction between differential expression genes (DEGs) was derived from the Search Tools for the Retrieval of Interacting Genes/Proteins (STRING) database. We obtained all interactions exhibiting a high confidence score (≥0.7) and visualised the protein-protein interaction map using Cytoscape 2.8. Genes were annotated to the KEGG pathway based on the online tool KEGG mapper (https://www.kegg.jp/kegg/mapper.html).

Statistical analysis

The resulting data were analysed using SPSS version 18.0 (IBM, Armonk, NY, USA). Quantitative results are presented as the mean and SD in tables. Paired comparisons of a mean number between age groups were performed using Student's t-test, assuming unequal variances. A chi-square test for independence was used for the comparison of proportions between age groups. Results were considered statistically significant at $P < 0.05$. For differentially expressed genes (DEGs) screening, edgeR package was used for exact test to normalise expression data and differential analysis. Significant DEGs were filtered with the relative fold change (≥1.5 or ≤0.67) under a p-value < 0.05.

3. Results

ICSI results and clinical outcomes are influenced by gestational age

A total of 448 oocytes were retrieved and 145 ART cycles were employed among 126 women. The detailed outcomes of ICSI and gestational of differential age group are reported in Table 2. Among 55 women aged ≤ 35 years, 193 of 216 (89.4 %) mature oocytes were successfully fertilised in-vitro; the cleavage patterns in dipronuclear (2PN) reached 98.4%. These rates were higher than for 37 women aged 36–40 years and 59 women aged > 40 years, but the difference was not statistically significant. However, the excellent embryo rate (58.9%) and blastocyst rate (55.8%) of the younger group were significantly higher than the older group (both 36 ~ 40 years old and > 40 years old).

Further analysis of the clinical outcomes found that the pregnancy rate (56.3%) of 96 transplanted cycles in the younger group was significantly higher than the rate (38.8%) of 49 transplanted cycles in the 36–40 years old group and the rate (5.9%) of 17 transplanted cycles in the >40 years old group. While the intima thickness and abortion rates had no significant difference amongst groups.

Table 2. ICSI results and clinical outcomes. (Data are the mean ± SD of each age group of patients. The asteriscs (*) marked in Older group represent significant differences ($p < 0.05$) when compared to Younger group.)

	Younger women (≤ 35 years old)	Older women (> 35 years old)	
		36~40 years old	> 40 years old
MII oocytes (n)	216	232	213
Fertilization rate (%)	193 (89.4)	178 (76.7)	184 (86.4)
2PN (%)	190 (98.4)	157 (88.2)	169 (91.8)
Top quality embryonic rate (%)	112 (58.9)	64 (40.8)*	86 (50.9)*
Blastocyst rate	106 (55.8)	51 (32.5)*	49 (29.0)*
ART cycles	96	49	17
Average intimal thickness	10.3	10.2	9.0
Number of embryos transferred (n)	1.7	1.8	1.5
Clinical pregnancy rate (%)	54 (56.3)	19 (38.8)*	1 (5.9)*
Abortion rate	7 (7.3)	6 (12.2)	1 (100.0)

Gene expression profile in CCs according to female age

For greater insight into the gene expression difference in CCs between younger and older ICSI patients, a comprehensive transcriptome analysis was performed under the Illumina HiSeq 2000 equipment. In total, 18,844 unigenes were significantly mapping to the NCBInr database. Furthermore, 193 unigenes were differentially expressed (94 up-regulated and 99 down-regulated) between the categories according to screen threshold with fold change ≥1.5 and significant p-value < 0.05. Detailed information of the top 20 up and down-regulated genes among older and younger females are listed in Table 3.

Table 3. Detailed information of top 20 up and down-regulated genes in comparison of Older/Younger.

Symbol	Ratio	p-value	Description	KEGG Orthology
CDH17	17.00	0.04	cadherin 17, LI cadherin (liver-intestine)	K06811
HAS1	15.50	0.02	hyaluronan synthase 1	K00752
IL7	14.50	0.01	interleukin 7	K05431
GAS6-AS1	12.83	0.01	GAS6 antisense RNA 1	K05464
LINC00861	11.00	0.01	long intergenic non-protein coding RNA 861	K11447;K00939;K00591
TERC	10.50	0.05	telomerase RNA component	-
RHOXF1	9.86	0.04	Rhox homeobox family, member 1	K09331;K09362
GNAT3	8.25	0.03	guanine nucleotide binding protein, alpha transducing 3	K04631
RGPD2	7.55	0.02	RANBP2-like and GRIP domain containing 2	K12172
IL18RAP	7.33	0.01	interleukin 18 receptor accessory protein	K05174
MAP2K6	7.00	0.03	mitogen-activated protein kinase kinase 6	K04433
RAB39A	6.33	0.04	RAB39A, member RAS oncogene family	K07924
HIST1H2BO	5.83	0.05	histone cluster 1, H2bo	K11252
TBX1	5.44	0.05	T-box 1	K10175
C2orf15	5.43	0.02	chromosome 2 open reading frame 15	K11447;K01539;K00939
FGF13	5.11	0.01	fibroblast growth factor 13	K04358
RDH12	4.57	0.01	retinol dehydrogenase 12 (all-trans/9-cis/11-cis)	K11153
GIPR	4.29	0.02	gastric inhibitory polypeptide receptor	K04580
C15orf48	4.08	0.00	chromosome 15 open reading frame 48	K03948
FAXC	3.80	0.05	failed axon connections homolog (Drosophila)	-
CLDN24	0.28	0.02	claudin 24	K06087

DDX11L2	0.28	0.04	DEAD/H (Asp-Glu-Ala-Asp/His) box helicase 11 like 2	K11273
CDH18	0.27	0.01	cadherin 18, type 2	K06805
NEIL3	0.26	0.04	nei endonuclease VIII-like 3 (E. coli)	K10569
PCDHA11	0.23	0.05	protocadherin alpha 11	K04602
POU5F1	0.23	0.02	POU class 5 homeobox 1	K09367
TMOD4	0.22	0.02	tropomodulin 4 (muscle)	K10370
ABHD11-AS1	0.20	0.04	long intergenic non-protein coding RNA 35	-
CA9	0.17	0.05	carbonic anhydrase IX	K01672
CDH1	0.17	0.00	cadherin 1, type 1, E-cadherin (epithelial)	K05689
RNF183	0.14	0.03	ring finger protein 183	-
LINC00240	0.14	0.02	long intergenic non-protein coding RNA 240	-
ANXA2P2	0.13	0.02	annexin A2 pseudogene 2	K09228;K00719;K01539;K00939;K00591
HLA-G	0.12	0.01	major histocompatibility complex, class I, G	K06751
SMCO3	0.11	0.03	single-pass membrane protein with coiled-coil domains 3	-
IGF2-AS	0.11	0.03	IGF2 antisense RNA	-
CLEC4GP1	0.11	0.04	C-type lectin domain family 4, member G pseudogene 1	K10061
FLJ46906	0.10	0.04	uncharacterized LOC441172	-
RNF144B	0.09	0.05	ring finger protein 144B	K11975
NPIPA3	0.05	0.04	nuclear pore complex-interacting protein-like 1-like	-

KEGG pathway analysis of DEGs

To identify the pathways that were altered in CCs related to female age, DEGs were annotated to the KEGG pathway based on the KEGG database. We classified the pathways based on the numbers of DEGs (Figure 1A). Differentially expressed genes are involved in cytokine-cytokine receptor interaction, PI3K-Akt signalling pathway, MAPK signalling pathway, Rap1 signalling pathway and cell adhesion molecules were highest counted. The visualized map of the PI3K-Akt pathway was derived to gain insight into the regulated genes (Figure 1B); they were mostly located in the plasma membrane receptors and the extracellular factors.

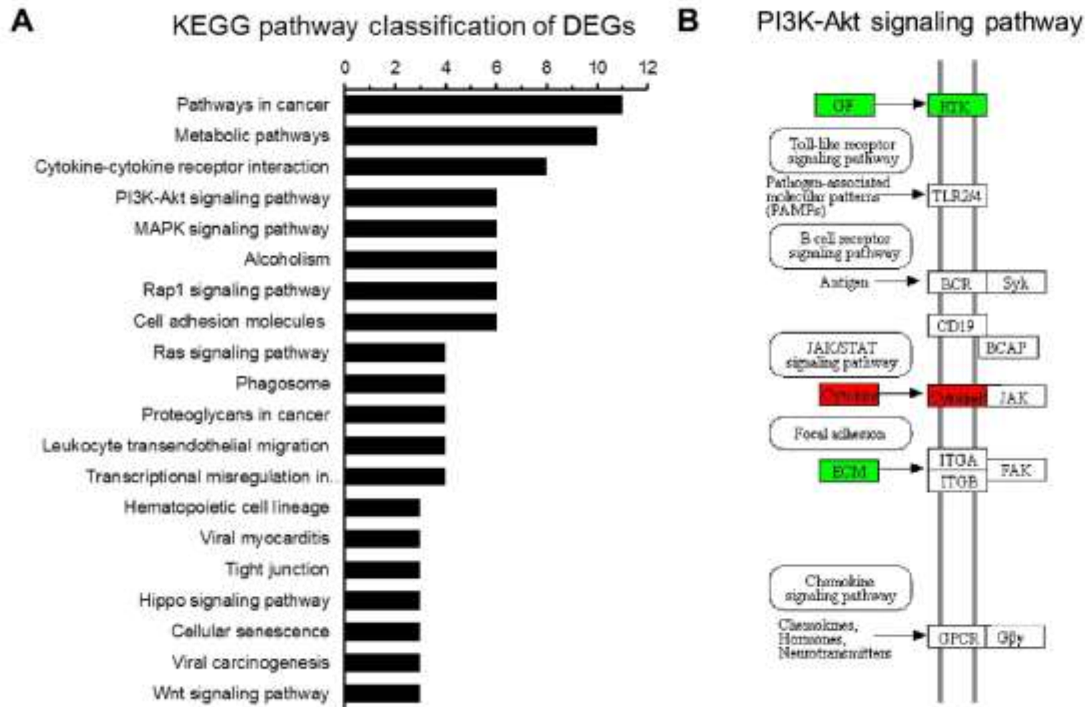

Figure 1. KEGG Pathway analysis of DEGs between older and younger groups. A: KEGG pathway classification of DEGs. B: Genes with significant change annotated to PI3K-Akt signaling pathway. Up-regulated genes were marked with red and down-regulated genes were marked with green.

DEGs significantly enriched in reproductive process in CCs of female age groups

The DEGs were annotated to GO categories according to the UniProt-GOA database. For biological process annotation, 64 unigenes were annotated to the developmental process, and 20 unigenes among all 134 annotated unigenes were related to the reproductive process. Gene products in the membrane and extracellular region were mostly enriched based on the cellular component annotation. In the molecular function category, gene products take part in binding, and catalytic activity obtained a high percentage, including DNA-binding transcription factor, signalling receptor and transmembrane transporter activities (Figure 2A). The genes annotated to several crucial GO biological process terms were classified and crosslinked as shown with a network in Figure 2B; down-regulated genes CDH1, KIT, VTN, PTCH1 and SOHLH2 and up-regulated genes A0A384MEK5, ICAM1 and TBX1 (up-regulated) were annotated to more than three enriched GO terms, which indicates the multi-functionality or significance of these genes.

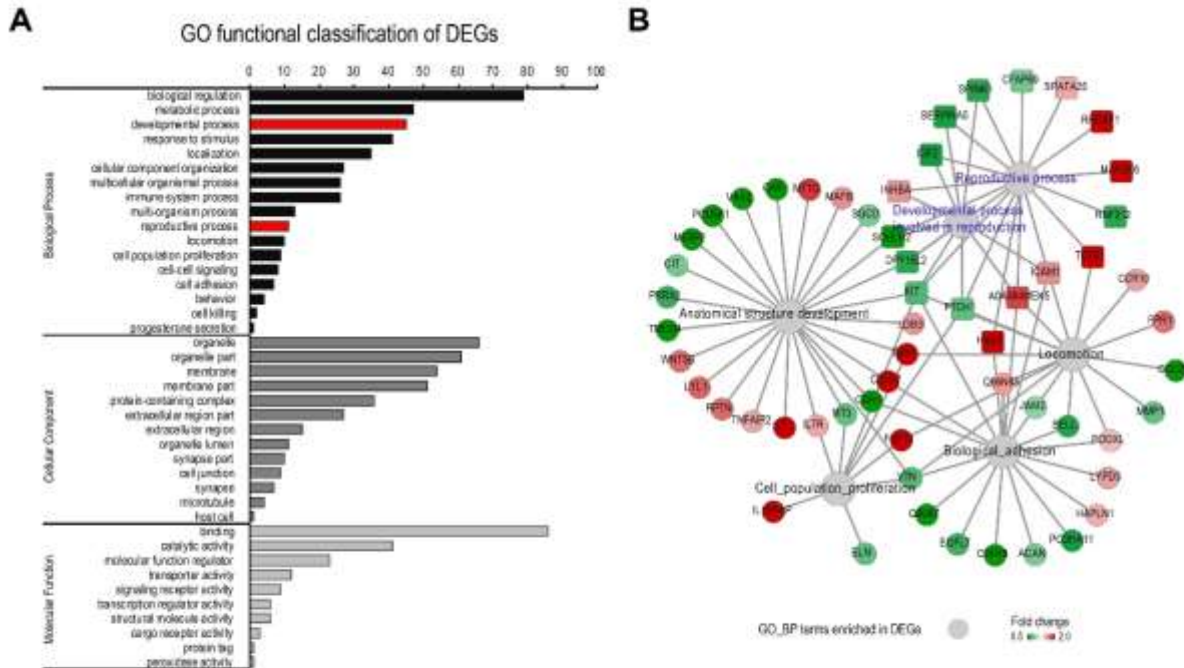

Figure 2. GO functional classification, enrichment and crosslink of DEGs. A: GO functional classification of DEGs. Crucial GO terms related with oocyte development were lighted in red. B: Crosslink of significantly enriched GO_BP terms of DEGs. The regulated level of genes (Older-vs-Younger) shows with different color. Up-regulated genes were colored with red and down-regulated with green.

Details and interaction network of DEGs enriched in the reproductive process

DEGs relative to reproduction were further classified into detailed functions (Figure 3A). Eight genes (RHOXF1, DPY19L2, CFAP69, KIT, SPINK1, SPATA25, SOHLH2, and SERPINA5) function in sexual reproduction or gamete generation, six genes (ICAM1, cKIT, PTCH1, IGF2, INHBA, SERPINA5) function in reproductive structure development and three genes (cKIT, DPY19L2, SPINK1) function in spermatid development. The heatmap of these reproductive-associated genes is illustrated in Figure 3B. The interaction network was established to identify key gene nodes included in the DEGs, especially those that participate in reproduction (Figure 3C). Candidate genes such as CTNNB1, CDH1, KIT, ICAM1, IGF2 had high degrees as key nodes; four of them (CDH1, KIT, ICAM1, IGF2) were annotated to the reproduction process.

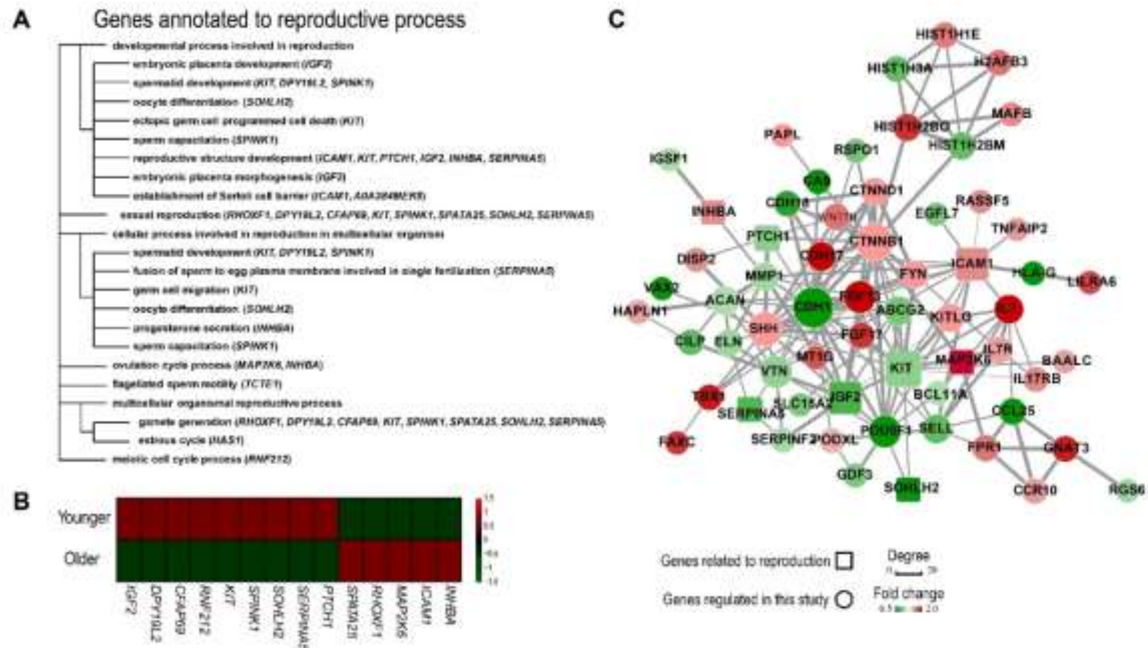

Figure 3. Details of reproductive related DEGs and the protein protein interaction network analysis of DEGs. A: The treemap of DEGs annotated to reproductive process. B: Heatmap of reproductive related DEGs. C, Protein-protein interaction network analysis of DEGs. The regulated level of genes (Older-vs-Younger) shows with different color. Up-regulated genes were colored with red and down-regulated with green. The protein related to reproduction were marked with rectangle and others with round.

Gene expression validation by qRT-PCR

In order to validate the RNA-seq data, the expression levels of five genes that show as the key nodes in the interaction network were checked using qRT-PCR. Three down-regulated genes (VTN, IGF2 and cKIT) and two up-regulated genes (ICAM1 and INHBA) in CCs derived from older females were confirmed and show consistently to the RNA-seq data (Figure 4). However, cKIT could not be amplified, possibly due to the low expression level in CCs.

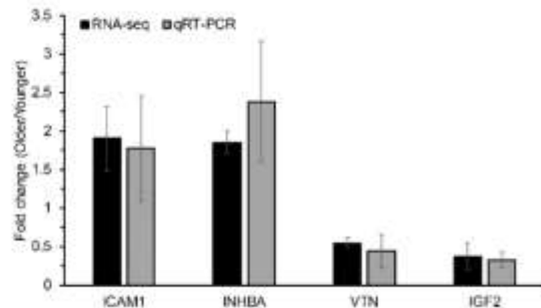

Figure 4. Relative expression level (fold change) of ICAM1, INHBA, VTN, and IGF2 in CCs derived from older females as compared to CCs derived from younger females. The black bars represent the expression level as detected by RNA-seq and white bars represent the gene expression as measured with qRT-PCR.

4. Discussions

The developing oocyte is surrounded by and interact with CCs. The overall health of oocyte could be revealed by biomarkers expressed in CCs. The non-invasive method for detecting CCs biomarkers can be used to calculate the transplanted embryos, whose competence will obtain the best quality of offspring (Kordus & LaVoie, 2017). Advanced reproductive age is a challenge for ART due to the functional disability of ageing ovaries.

Previous studies have demonstrated that oocytes and their surrounding CCs from older females (>35 years) may increase mitochondrial DNA instability (May-Panloup et al., 2016) and oxidative damage (Mihalas et al., 2017). Gene expression profile of CCs has been previously used for screening markers of oocyte quality (Ola & Sun, 2012). Pourret et al. (2016) summarised the biomarkers of the CCs of previous studies. In this study, we performed an RNA-seq analysis to identify the differentially expressed genes related to age in CCs derived from different age women (≤35 years of age and >35 years of age); there was no significant difference in clinical characteristics in each age group, and the top quality embryonic rate, blastocyst rate and clinical pregnancy rate in the younger group were significantly higher than the older group. In total, 18,844 unigenes significantly mapped to the NCBInr database. Furthermore, 193 unigenes

were differentially expressed (94 up-regulated and 99 down-regulated) between the two female age categories. Many earlier gene microarray results evaluated the transcriptomes of human cumulus cells between women among different ages (divided in age 35, younger or older) (Al-Edani et al., 2014; Hurwitz et al., 2010; Kordus & LaVoie, 2017). However, some changes have not been validated through qRT-PCR, thereby limiting data interpretation (Chin et al., 2002). RNA-seq can provide more information that is absent in the microarray method at the transcriptional level. In RNA-seq, the cDNA was under sequencing deeply to detect the transcripts. So far, RNA sequencing data regarding CCs in females of AMA are limited. In our study, the DEGs identified by RNA-seq in CCs grouped by age from 126 patients were annotated to GO categories according to the UniProt-GOA database; the DEGs related to reproduction were further classified to detailed functions. Genes function as sexual reproduction, gamete generation, reproductive structure development and spermatid development were found changed in different age groups. Maternal age affects differential mRNA gene expression in CCs (Al-Edani et al., 2014; Hurwitz et al., 2010; Kedem et al., 2014; Lee et al., 2010; McReynolds et al., 2012). The perspective of this study was to assess whether reproductive ageing is accompanied by differential gene expression in CCs from in-vitro fertilization (IVF) patients. Notably, we found several interesting genes (IGF2, INHBA and SERPINA5), with down-regulation of IGF2 and SERPINA5 and up-regulation of INHBA the AMA group. Those genes are involved in embryonic placenta development (IGF2), reproductive structure development (IGF2, INHBA, SERPINA5), embryonic placenta morphogenesis (IGF2), sexual reproduction (SERPINA5), fusion of sperm to egg plasma membrane in single fertilization (SERPINA5), progesterone secretion (INHBA), ovulation cycle process (INHBA) and gamete generation (SERPINA5).

The differentially expressed genes (DEGs) of luteinized granular cells (GCs) were explored by Hurwitz et al. (2010). In their study, the patients under treatment of IVF were divided into two groups: the younger group (≤30) and the older group (≥40). As a result, they identified 120 DEGs; in the older group, they found that three up-regulated and 117 down-regulated genes were expressed in GCs. Finally, they suggested that the down-regulation of IL-1 and IL-6 gene families -in GCs were related to age (Hurwitz et al., 2010).

In another study, Al-Edani et al. (2014) detected genes related to advanced age by the microarray method from three age groups. They found that many angiogenic genes, such as ANGPTL4, LEPR, TGFBR3 and FGF2, were up-regulated in the CCs of older females. In the patients within the age range of 31 and 36, the genes involved in the TGF-β signalling pathway, including AMH, TGFB1, inhibin and activin receptor, were down-regulated in CCs; the genes involved in the insulin signalling pathway, including IGFBP3, PIK3R1 and IGFBP5 were up-regulated in CCs

A proteomic analysis of CCs was performed by McReynolds et al. (2012). There were two groups in their experimental design: the younger fertile female donors (n=40) and the older infertile females (n = 48). They detected 1,432 proteins expressed in cumulus cells; 110 proteins were DEPs (>1.5-fold change) which were associated with ageing in women. The annotation of the pathway for these DEPs revealed that many pathways are involved in the ageing of the oocyte, including metabolism (ACAT2, HSD17B4, ALDH9A1, MVK, CYP11A1 and FDFT1), oxidative phosphorylation (OP; NDUFA1, UQCRC1, MT-ATP6, ATP5I and MT-ATP8) and post-transcriptional mechanisms (KHSRP, SFPQ, DDX46, SNRPF, ADAR, NHPL1 and U2AF2) (McReynolds et al., 2012).

At the transcriptional level, our data revealed seven genes (CTNNB1, ICAM1, INHBA, SERPINA5, CDH1, KIT and IGF2) that might relate to advanced maternal age and impact the molecular signature of cumulus cells. In those genes, CTNNB1, ICAM1 and INHBA mRNA expression were shown to be up-regulated in CCs isolated from oocytes in the older group; in contrast, SERPINA5, CDH1, KIT, and IGF2 expression levels were down-regulated. Those changes of genes expressed in CCs suggest that the ageing of eggs not only affects the expression of their genes but also affects the expression of genes in CCs.

The protein (Cadherin-Associated Protein, Beta 1) encoded by CTNNB1 and involved in adherens junctions (AJs), which is a complex of many proteins. AJs regulates cell growth and adhesion among cells to achieve the maintenance and creation of epithelial cell layers (Kafri et al., 2016; Morikawa et al., 2011). In cell adhesion, CTNNB1 is a component of E-cadherin (catenin adhesion complex) that consists of beta-catenin/CTNNB1, E-cadherin/CDH1 and alpha-catenin/CTNNA1; this complex is involved in AJs (Chan et al., 2002; Chen et al., 2018). Similarly, Intercellular Adhesion Molecule 1 encoded by ICAM1 is also involved in intercellular adhesion. In our study, CTNNB1 and ICAM1 were both up-regulated in the advanced age group, which reveals that ageing may affect the adhesion between the oocyte and cumulus cells. Notably, CDH1 is a classical cadherin of the cadherin superfamily and is a calcium-dependent cell-cell adhesion protein. An important paralog of this gene is CDH3. The protein encoded by CDH3 is involved in tight junctions after the activation of the oocyte in bovines (Chen et al., 2018).

INHBA (Inhibin Subunit Beta A) is a member of the TGF-β (transforming growth factor-beta) superfamily which activates and inhibits to stimulate hormone secretion from the pituitary gland (Thompson et al., 2005). GDF-9 and BMP-15, which are the factors secreted by oocytes, are also involved in the TGF-beta superfamily. The absence of GDF-9 and BMP-15 in females has been shown to cause sterility (Dong et al., 1996; Galloway et al., 2000). The expression levels of GDF9 and BMP15 affects many aspects of reproduction, including positive pregnancy, mature oocytes, fertilization rate and embryo quality (Li et al., 2014). In our study, INHBA mRNA expression was up-regulated in CCs of the older age groups, in contrast to GDF-9 and BMP-15, which may indicate that INHBA is related to negative pregnancies after embryo transfer.

SERPINA5 is in the serpin family and is an inhibitor of serine proteases. SERPINA5 influences reproduction through serine proteases of seminal plasma (Espana et al., 1991; He et al., 1999). It also regulates the motility and fertilization of sperm; when spermatozoa transfer from the male reproductive tract to the female reproductive tract, the semenogelin degrade (Kise et al., 1996). In our study, SERPINA5 was down-regulated in the AMA group; this suggests that the expression of SERPINA5 affects the function of sperm and CCs.

KIT is a transmembrane receptor of mast cell growth factor (MGF). It activates the AKT1 signalling pathway by phosphorylation of PIK3R1, which is the regulatory subunit of phosphatidylinositol 3-kinase. IGF2 is also a growth factor and a member of the insulin family of polypeptide growth factors. As current literature reports, the growth factor relates to development and growth (Alvino et al., 2011). Notably, IGF2 is a paternally imprinted gene that governs normal embryonic development (Ratajczak, 2012). In our study, KIT and IGF2 were down-regulated in the AMA group; this suggests that the activities of growth factors expressed in CCs decrease with the ageing of the oocyte.

Our study reveals that the molecular characteristics of cumulus cells at transcription level are impacted by advanced ageing in females.

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References

1. Al-Edani, T., Assou, S., Ferrieres, A., Bringer Deutsch, S., Gala, A., Lecellier, C. H., Ait-Ahmed, O., & Hamamah, S. (2014). Female aging alters expression of human cumulus cells genes that are essential for oocyte quality. Biomed Research International, 2014, 964614.
2. Alvino, C. L., Ong, S. C., McNeil, K. A., Delaine, C., Booker, G. W., Wallace, J. C., & Forbes, B. E. (2011). Understanding the mechanism of insulin and insulin-like growth factor (IGF) receptor activation by IGF-II. PloS One, 6(11), e27488.
3. Assou, S., Haouzi, D., De Vos, J., & Hamamah, S. (2010). Human cumulus cells as biomarkers for embryo and pregnancy outcomes. Molecular Human Reproduction, 16(8), 531-538.
4. Bhattacharya S. (2003). Effective Use of Assisted Reproduction. Human Ferility, 6 Suppl 1:S60-2.
5. Chan, T. A., Wang, Z., Dang, L. H., Vogelstein, B., & Kinzler, K. W. (2002). Targeted inactivation of CTNNB1 reveals unexpected effects of beta-catenin mutation. Proceedings of the National Academy of Sciences of the United States of America, 99(12), 8265-8270.
6. Suneeta, M., Lakhbir, D., Sanjeev, S., Sanjay, C., Garg, B.S., Singh, N. (2012). Infertility. International Journal of Gynecology & Obstetrics. 2012:508276.
7. Chen, F., Fu, Q., Pu, L., Zhang, P., Huang, Y., Hou, Z., Xu, Z., Chen, D., Huang, F., Deng, T., Liang, X., Lu, Y., & Zhang, M. (2018). Integrated Analysis of Quantitative Proteome and Transcriptional Profiles Reveals the Dynamic Function of Maternally Expressed Proteins After Parthenogenetic Activation of Buffalo Oocyte. Molecular & Cellular Proteomics, 17(10), 1875-1891.
8. Chin, K. V., Seifer, D. B., Feng, B., Lin, Y., & Shih, W. C. (2002). DNA microarray analysis of the expression profiles of luteinized granulosa cells as a function of ovarian reserve. Fertility and Sterility, 77(6), 1214-1218.

9. David Lindsay Healy, A. O. T., Anders Nyboe Andersen. (1994). Female infertility: causes and treatment. Lancet., 343(8912), 1539-1544.
10. Dong, J., Albertini, D. F., Nishimori, K., Kumar, T. R., Lu, N., & Matzuk, M. M. (1996). Growth differentiation factor-9 is required during early ovarian folliculogenesis. Nature, 383(6600), 531-535.
11. Espana, F., Gilabert, J., Estelles, A., Romeu, A., Aznar, J., & Cabo, A. (1991). Functionally active protein C inhibitor/plasminogen activator inhibitor-3 (PCI/PAI-3) is secreted in seminal vesicles, occurs at high concentrations in human seminal plasma and complexes with prostate-specific antigen. Thrombosis Research, 64(3), 309-320.
12. Fragouli, E., Wells, D., Iager, A. E., Kayisli, U. A., & Patrizio, P. (2012). Alteration of gene expression in human cumulus cells as a potential indicator of oocyte aneuploidy. Human Reproduction, 27(8), 2559-2568.
13. Galloway, S. M., McNatty, K. P., Cambridge, L. M., Laitinen, M. P., Juengel, J. L., Jokiranta, T. S., McLaren, R. J., Luiro, K., Dodds, K. G., Montgomery, G. W., Beattie, A. E., Davis, G. H., & Ritvos, O. (2000). Mutations in an oocyte-derived growth factor gene (BMP15) cause increased ovulation rate and infertility in a dosage-sensitive manner. Nature Genetics, 25(3), 279-283.
14. Harrison, B. J., Hilton, T. N., Riviere, R. N., Ferraro, Z. M., Deonandan, R., & Walker, M. C. (2017). Advanced maternal age: ethical and medical considerations for assisted reproductive technology. International Journal of Womens Health, 9, 561-570.
15. He, S., Lin, Y. L., & Liu, Y. X. (1999). Functionally inactive protein C inhibitor in seminal plasma may be associated with infertility. Molecular Human Reproduction, 5(6), 513-519.
16. Huang, Z., & Wells, D. (2010). The human oocyte and cumulus cells relationship: new insights from the cumulus cell transcriptome. Molecular Human Reproduction, 16(10), 715-725.
17. Hurwitz, J. M., Jindal, S., Greenseid, K., Berger, D., Brooks, A., Santoro, N., & Pal, L. (2010). Reproductive aging is associated with altered gene expression in human luteinized granulosa cells. Reproductive Sciences, 17(1), 56-67.
18. Resolve. (n.d.). Risk factors: Risk factors for women. Subtitle.
19. Iager, A. E., Kocabas, A. M., Otu, H. H., Ruppel, P., Langerveld, A., Schnarr, P., Suarez, M., Jarrett, J. C., Conaghan, J., Rosa, G. J. M., Fernandez, E., Rawlins, R. G., Cibelli, J. B., & Crosby, J. A. (2013). Identification of a novel gene set in human cumulus cells predictive of an oocyte's pregnancy potential. Fertility and Sterility, 99(3), 745-752.
20. Jackson, S., Hong, C., Wang, E. T., Alexander, C., Gregory, K. D., & Pisarska, M. D. (2015). Pregnancy outcomes in very advanced maternal age pregnancies: the impact of assisted reproductive technology. Fertility and Sterility, 103(1), 76-80.
21. Jia, B. Y., Xiang, D. C., Quan, G. B., Zhang, B., Shao, Q. Y., Hong, Q. H., & Wu, G. Q. (2019).
22. Transcriptome analysis of porcine immature oocytes and surrounding cumulus cells after vitrification and in vitro maturation. Theriogenology, 134, 90-97.
23. Kafri, P., Hasenson, S. E., Kanter, I., Sheinberger, J., Kinor, N., Yunger, S., & Shav-Tal, Y. (2016). Quantifying beta-catenin subcellular dynamics and cyclin D1 mRNA transcription during Wnt signaling in single living cells. Elife, 5.
24. Kedem, A., Yung, Y., Yerushalmi, G. M., Haas, J., Maman, E., Hanochi, M., Hemi, R., Orvieto, R., Dor, J., & Hourvitz, A. (2014). Anti Mullerian Hormone (AMH) level and expression in mural and cumulus cells in relation to age. J Ovarian Res, 7, 113.
25. Kise, H., Nishioka, J., Kawamura, J., & Suzuki, K. (1996). Characterization of semenogelin II and its molecular interaction with prostate-specific antigen and protein C inhibitor. European Journal of Biochemistry, 238(1), 88-96.
26. Kordus, R. J., & LaVoie, H. A. (2017). Granulosa cell biomarkers to predict pregnancy in ART: pieces to solve the puzzle. Reproduction, 153(2), R69-R83.
27. Lee, M. S., Liu, C. H., Lee, T. H., Wu, H. M., Huang, C. C., Huang, L. S., Chen, C. M., & Cheng, E. H. (2010). Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells. Journal of Assisted Reproduction and Genetics, 27(11), 629-639.
28. Li, Y., Li, R. Q., Ou, S. B., Zhang, N. F., Ren, L., Wei, L. N., Zhang, Q. X., & Yang, D. Z. (2014). Increased GDF9 and BMP15 mRNA levels in cumulus granulosa cells correlate with oocyte maturation, fertilization, and embryo quality in humans. Reproductive Biology and Endocrinology, 12, 81.
29. Liu, Q., Zhang, J., Wen, H., Feng, Y., Zhang, X., Xiang, H., Cao, Y., Tong, X., Ji, Y., & Xue, Z. (2018). Analyzing the Transcriptome Profile of Human Cumulus Cells Related to Embryo Quality via RNA Sequencing. Biomed Research International, 2018, 9846274.
30. May-Panloup, P., Boucret, L., Chao de la Barca, J. M., Desquiret-Dumas, V., Ferre-L'Hotellier, V., Moriniere, C., Descamps, P., Procaccio, V., &

Reynier, P. (2016). Ovarian ageing: the role of mitochondria in oocytes and follicles. Human Reproduction Update, 22(6), 725-743.

31. McReynolds, S., Dzieciatkowska, M., McCallie, B. R., Mitchell, S. D., Stevens, J., Hansen, K., Schoolcraft, W. B., & Katz-Jaffe, M. G. (2012). Impact of maternal aging on the molecular signature of human cumulus cells. Fertility and Sterility, 98(6), 1574-1580 e1575.
32. Mihalas, B. P., Redgrove, K. A., McLaughlin, E. A., & Nixon, B. (2017). Molecular Mechanisms Responsible for Increased Vulnerability of the Ageing Oocyte to Oxidative Damage. Oxidative Medicine and Cellular Longevity, 2017, 4015874.
33. Molinari, E., Bar, H., Pyle, A. M., & Patrizio, P. (2016). Transcriptome analysis of human cumulus cells reveals hypoxia as the main determinant of follicular senescence. Molecular Human Reproduction, 22(8), 866-876.
34. Morikawa, T., Kuchiba, A., Yamauchi, M., Meyerhardt, J. A., Shima, K., Nosho, K., Chan, A. T., Giovannucci, E., Fuchs, C. S., & Ogino, S. (2011). Association of CTNNB1 (beta-catenin) alterations, body mass index, and physical activity with survival in patients with colorectal cancer. JAMA, 305(16), 1685-1694.
35. O'Brien, Y. M., Ryan, M., Martyn, F., & Wingfield, M. B. (2017). A retrospective study of the effect of increasing age on success rates of assisted reproductive technology. International Journal of Gynecology & Obstetrics, 138(1), 42-46.
36. Ola, S. I., & Sun, Q. Y. (2012). Factors influencing the biochemical markers for predicting mammalian oocyte quality. Journal of Reproduction and Development, 58(4), 385-392.
37. Pantos, K., Athanasiou, V., Stefanidis, K., Stavrou, D., Vaxevanoglou, T., & Chronopoulou, M. (1999). Influence of advanced age on the blastocyst development rate and pregnancy rate in assisted reproductive technology. Fertility and Sterility, 71(6), 1144-1146.
38. Pourret, E., Hamamah, S., & Ait-Ahmed, O. (2016). [Biomarkers of the cumulus cells in medically assisted procreation: State-of-the-art]. Gynécologie, Obstétrique & Fertilité, 44(11), 647-658.
39. Ratajczak, M. Z. (2012). Igf2-H19, an imprinted tandem gene, is an important regulator of embryonic development, a guardian of proliferation of adult pluripotent stem cells, a regulator of longevity, and a 'passkey' to cancerogenesis. Folia Histochemica et Cytobiologica, 50(2), 171-179.
40. Rooney, K. L., & Domar, A. D. (2018). The relationship between stress and infertility. Dialogues in Clinical Neuroscience, 20(1), 41-47.
41. Sauer, M. V. (2015). Reproduction at an advanced maternal age and maternal health. Fertility and Sterility, 103(5), 1136-1143.
42. Thompson, T. B., Lerch, T. F., Cook, R. W., Woodruff, T. K., & Jardetzky, T. S. (2005). The structure of the follistatin:activin complex reveals antagonism of both type I and type II receptor binding. Developmental Cell, 9(4), 535-543.
43. Wathlet, S., Adriaenssens, T., Segers, I., Verheyen, G., de Velde, H. V., Coucke, W., El, R. R., Devroey, P., & Smitz, J. (2011). Cumulus cell gene expression predicts better cleavage-stage embryo or blastocyst development and pregnancy for ICSI patients. Human Reproduction, 26(5), 1035-1051.
44. Yerushalmi, G. M., Salmon-Divon, M., Yung, Y., Maman, E., Kedem, A., Ophir, L., Elemento, O., Coticchio, G., Dal Canto, M., Mignini Renzinu, M., Fadini, R., & Hourvitz, A. (2014). Characterization of the human cumulus cell transcriptome during final follicular maturation and ovulation. Molecular Human Reproduction, 20(8), 719-735.

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