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ASSISTED REPRODUCTION

Association of creatin kinase B and peroxiredoxin 2 expression with age and embryo quality in cumulus cells

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Abstract
Purpose The purpose of this study was to identify age-related oocyte or embryo markers suitable for non-invasive analysis, as women over 38 years of age experience diminished pregnancy and ovulation rates.
Methods We used real-time quantitative PCR to examine the gene expression profiles in cumulus cells acquired from older and younger age groups. We selected 11 genes involved in three functions that directly affect cellular aging: cell cycle control, apoptosis, and metabolism.
Results *CKB* and *PRDX2* were up-regulated in women older than 38 years, and the expression of these genes in cumulus cells was associated with embryo quality. In good-quality embryos, *CKB* expression was higher in the cumulus cells acquired from both older and younger age groups than in poor-quality embryos.
Conclusions These potential relationships among cumulus cell gene expression, oocyte quality, and age may expand our understanding of oogenesis and embryo development. *CKB* and *PRDX2* may serve as biomarkers or therapeutic targets for the developmental potential of oocytes.

Keywords Aging · Creatin kinase B (CKB) · Cumulus cells · Embryo quality · Peroxiredoxin 2 (PRDX2)

Capsule In cumulus cell gene expression, Creatin kinase B and peroxiredoxin 2 may serve as biomarkers or therapeutic targets for the developmental potential of oocytes.

Where the work was done: Division of Infertility Clinic, Lee Women's Hospital, Taichung, Taiwan.

Introduction

Most in vitro fertilization (IVF) clinics rely on noninvasive examination of developmental and morphological aspects of oocytes and in vitro cultured embryos for embryo selection [1–3]. Since cumulus cells surround the oocyte

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inside the follicle, a close relationship or communication between cumulus cells and the oocyte has been proposed. Indeed, the role of the surrounding cumulus cells in oocyte maturation, ovulation, and fertilization has been extensively studied [4–6], yet little is known about their contributions to oocyte aging.

Mammalian oocyte development is coordinated with follicular development and complex interactions with granulosa cells [7]. Granulosa cells have been shown to be involved in the regulation of murine ovarian oocyte metabolism and maturation [8], supporting the concept that oocyte development is dependent upon communication with cumulus cells. Mural granulosa cells are associated with the follicle wall, while the cumulus cell population is associated with the oocyte. Oocytes control their environment by suppressing differentiation of the mural granulosa cell phenotype and promoting differentiation of the cumulus cell phenotype through secretion of labile paracrine signaling factors [9]. Cumulus expansion plays an essential role in normal oocyte development, ovulation, and fertilization, as well as embryo development and implantation [10, 11]. Errors in this regulatory mechanism may result in the production of oocytes unable to undergo embryo development.

The follicle pool declines exponentially with age, and begins to disappear at a marked rate approximately at age 37–38 [12, 13]; along the same lines, IVF for women over 38 years of age was not as efficient as for women younger than 30 years old [14]. Alterations in cumulus expansion may occur as women age and may be responsible for their reproductive disadvantage [15]. As most multicellular organisms or cells age, gene expression profiles change; therefore, a comparative molecular analysis of follicles, such as the cumulus cells or the oocytes derived from younger and older age groups, may generate information necessary for a better understanding of embryogenesis. This process may also aid in the identification of age-related factors of biological capacity of women, reflecting the developmental potential of their gametes and their biological environments.

In order to elucidate the interplay between cumulus cells and embryogenesis during the process of oocyte aging, we used microarrays to investigate the associations among the expression profiles in older and younger women. Eleven cumulus cell genes were selected for further study based on their putative aging functions as well as on the results of the microarray analysis. The expression of selected cumulus cell genes related to cell cycle, apoptosis, and metabolism were surveyed in cells acquired from older and younger women, and relationships with embryo quality and subject age were investigated in an effort to elaborate the processes underpinning embryo development.

Materials and methods

Collection of cumulus cells from IVF patients

Complete institutional review board approval and written consents were obtained from the 44 patients prior to the retrieval of cumulus cells. For microarray analysis, we collected oocyte-cumulus complexes from six donors: three young patients (≦28 years old) and three older patients (≧38 years old). The relationships between embryo quality and gene expression were evaluated from another 38 patients undergoing IVF programs in the Division of Infertility Clinic, Lee Womens' Hospital, Taichung, Taiwan. A total of 178 embryos were collected for study on day 3 after in vitro insemination and culture. Analyzed embryos (a subset from each patient) were selected on a random basis.

Controlled ovarian stimulation

The female partner of the infertile couples participating in this study followed a stimulation protocol that began with daily subcutaneous injections of leuprolide acetate (LA Lupron, Takeda Pharmaceutics, Germany), 0.1 mg on day 21 of their pre-stimulation cycle (long protocol); the injections were continued until human chorionic gonadotrophin (hCG) was administered. Subcutaneous gonadotropin administration (Gonal-F; Serono, Bari, Italy), 225 IU/day, was performed to stimulate follicular development. The resulting ovarian response was monitored by transvaginal ultrasound and serum estradiol levels. When two or more follicles reached a maximum diameter of 18 mm and the serum estradiol levels exceeded 600 pg/ml, 10,000 IU hCG (Profasi; Serono) was administered. Transvaginal oocyte retrieval was performed 32–34 h after hCG injection. The cumulus cells were removed from the oocyte, washed separately with phosphate-buffered saline (PBS, Invitrogen Corp, Carlsbad, CA, USA), and total RNA or mRNA was extracted.

Mature oocytes were identified by the presence of the first polar body after removal of the cumulus cells in the IVF cycles at the fertilization check, which was performed 16–18 h after insemination. Unfertilized oocytes and diploid zygotes (2PN) were identified and counted during the fertilization check. Diploid zygotes were cultured individually into 15 μl microdroplets of G2.1 medium (Scandinavian IVF Science/Vitrolife, Gothenburg, Sweden) overlaid with 2.5 ml mineral oil in Falcon 3002 culture dishes (Becton Dickinson Labware, Franklin Lakes, NJ, USA). Embryo developmental states were recorded from day 0 to day 3.

Microarray with fluorescence detection

We collected and pooled 12 oocyte-cumulus complexes from three young patients (<28 years old) and 12 oocyte-

cumulus complexes from three older patients (>38 years old). All oocyte-cumulus complexes were separated by fire-polished solid glass pipette and removed to tubes. Total RNA was isolated with TRIzol (Life Technologies, Rockville, MD, USA) from cumulus samples of three patients undergoing IVF and loaded onto a Qiagen RNeasy column (Qiagen Inc, Valencia, CA, USA) for purification according to the manufacturer's instructions. Electrophoresis on a 1% agarose formaldehyde gel was utilized to determine the integrity of the RNA preparation. Total RNA (1.5–3 μg) was reverse transcribed with Superscript II RNase H-reverse transcriptase (Gibco BRL, Long Island, NY, USA) to generate Cy3- and Cy5-labeled complementary DNA (cDNA) probes. The labeled probes were hybridized to the ABC Human UniversoChip 20 (eGenomix Technology Corp, Taipei, Taiwan), a commercial cDNA microarray containing 19,844 immobilized cDNA fragments. Fluorescence intensities of Cy3 and Cy5 targets were measured and scanned separately using a GenePix 4000B Array Scanner (Axon Instruments, Molecular Devices Corp, Sunnyvale, CA, USA) and eGenomix V1.0 software (eGenomix). The results were normalized, first to detect the labeling efficiencies of the two fluorescent dyes, then to determine differential gene expression between samples from older and younger women.

Microarray data analyses

Differentially expressed genes were identified when the absolute value of the Cy5/Cy3 was greater than two-fold (up-regulated) or less than 0.5-fold (down-regulated), and the signal value of the fluorescence intensity of either Cy3 or Cy5 were greater than 1,000.

Gene selection and real-time quantitative PCR

Gene expression profiles were determined from the cumulus cells acquired from women either older than 38 years of age or younger than 28 years of age (see above). The expression of genes whose functional roles encompassed cell cycle regulation, metabolism, and apoptosis were studied. Of these genes, 11 varied dependent on the subject's age, and thus were considered as candidate genes for further study (Table 1).

Messenger RNA was extracted with the Dynabeads mRNA DIRECT Kit (Dynal Biotech ASA, Oslo, Norway) according to the manufacturer's protocol. Briefly, we added 120 μl Lysis/Binding Buffer to the cumulus cells and inverted the tube repeatedly to obtain complete lysis. We transferred the lysate to a tube containing 10 μl Dynabeads Oligo (dT) 25. The beads were mixed with the sample lysate and incubated with continuous mixing for 3–5 min at room temperature to allow the poly (A) tail of the mRNA to anneal to the Oligo-dT on the beads. This vial was placed on a magnet for 2 min, and then the supernatant was removed. The bead/mRNA complexes were washed twice with 240 μl Washing Buffer A and once with 120 μl Washing Buffer B at room temperature. The magnet was used to separate the beads from the solution between each washing step. The beads containing mRNA were then ready for reverse transcription into cDNA.

The following reagents were added to a tube containing the bead/mRNA complex for a final reaction volume of 30 μl: 17 μl diethyl pyrocarbonate (DEPC)-treated water, 1 μl RNaseOUT recombinant RNase inhibitor (40 U/μl, Invitrogen), 1 μl dNTPs (10 mM each dATP, dGTP, dCTP, and dTTP), 1 μl Oligo (dT) 20 primer (50 μM, Invitrogen), 4 μl of 5X First-Strand Buffer (Invitrogen), 2 μl DTT (0.1 M), and 1 μl SuperScript III Reverse Transcriptase (200 U/μl). The reaction was carried out at 50°C for 1 h, 70°C for 15 min, 4°C for 2 min, and 37°C for 20 min. The resulting single-stranded cDNA was used as template for quantitative real-time polymerase chain reaction (qPCR).

Plasmid DNA previously cloned from the PCR product corresponding to β-actin cDNA (primers Forward-1 and Reverse-1, Table 2) was used as a standard. Standard DNA was amplified in duplicate as 5- or 10-fold serial dilutions. A reaction mixture with no template cDNA served as the negative control for each gene.

Reaction volumes of 25 μl contained 1 μl cDNA, 12.5 μl 2x HotSybr PCR Reaction Mix (NuStar Laboratory, USA), 5 μl each gene-specific primer (0.2–0.5 μM, depending on the gene), and 6.5 μl sterile distilled water. Primers used in this study are listed in Table 2. The tubes were subjected to thermal cycling using the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The amplification program consisted of heating at 95°C for 5 min, followed by 95°C for 5 s and 60°C for 30 s for 50 cycles. Each cumulus sample was tested in triplicate for each gene in this study. The amplification curve is shown in Fig. 1. We added a dissociation stage to determine whether there was any contamination.

Embryo grading

To analyze the relationship between embryo quality and gene expression in cumulus cells, we characterized cleavage-stage embryos on day 3. Embryos were graded according to previously described criteria [16, 17], with a slight modification. Grade 1 embryos were considered to be top-quality embryos, with evenly shaped blastomeres, uniform cytoplasms, and no visible cytoplasmic fragments. Grade 2 embryos displayed either unequal blastomeres or cytoplasmic fragments taking up less than 20% of the embryonic perivitelline space. Grade 3 embryos were comprised of 20–50% cytoplasmic fragments by volume,

Table 1 The 11 genes with age-dependent differential expression, as quantified by microarray analysis

Gene function	Gene name	Gene symbol	Up or down regulation in the older group
Cell cycle	Cullin 1	CUL1	up
	Cysteine-rich-angiogenic inducer-61	CYR61	up
	Metallothionein 1E	MT1E	up
	Creatine kinase B	CKB	up
	Cyclin-dependent kinase 4	CDK4	up
	Adducin 3 (gamma)	ADD3	down
Apoptosis	Immediate early response 3	IER3	up
	*Cullin 1	CUL1	up
	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	up
	*Cyclin-dependent kinase 4	CDK4	up
Metabolism	Peroxiredoxin 2	PRDX2	up
	Ribosomal protein S28	RPS28	up
	*Creatine kinase	CKB	up
	Procollagen-proline, 2-oxoglutarate (proline 4-hydroxylase)-beta polypeptide	P4HB	up

*Repeat gene in this list

Table 2 Primer sets used for quantitative real-time PCR

Gene		Primer sequence (5′ to 3′)
β-actin	Forward-1	CTGAGGCACTCTTCCAGCCTT
	Reverse-1	CACATCTGCTGGAAGGTGGAC
	Forward	CTGGCACCCAGCACAATG
	Reverse	GCCGATCCACACGGAGTACT
ADD3	Forward	CAGTCTTGGCATGGTCACACCT
	Reverse	CTACAAGTCTGTACAGGCTGGC
CDK4	Forward	CTTGCCAGCCGAAACGA
	Reverse	GATGCAATTGGCATGAAGGAA
CKB	Forward	GATACTACGCGCTCAAGAGCA
	Reverse	TCTCATTGTGCCAGATACCG
CUL1	Forward	ACAATGACGCTGGCTTTGTG
	Reverse	CTTGGTAACCGCGTTGTTGTT
CYR61	Forward	GAATTGATTGCAGTTGGAAAAGG
	Reverse	GTATAGGATGCGAGGCTCCATT
FOSB	Forward	GTCTGGAGTTTGTGCTGGTGG
	Reverse	CTTCCTTAGCCGGTGCTGAGC
IER3	Forward	GAGCCTCGACCTGACCTGTCT
	Reverse	TGGCTGGGTTCGGTTCCT
MT1E	Forward	TCCTGCAAGTGCAAAGAGTGC
	Reverse	AGAAGGATGCACTCAGGG
P4HB	Forward	TGCACAGCTTCCCCACACT
	Reverse	CGTTCCCCGTTGTAATC
PRDX2	Forward	GTCCTTCGCCAGATCACTGTTA
	Reverse	CATGCTCGTCTGTGTACTGGAAGGC
RPS28	Forward	GAATTCATGGACGACACGAG
	Reverse	GACATCCAAGACCCAGCGAG

and in Grade 4 embryos, cytoplasmic fragments made up more than 50% of the perivitelline space.

According to the developmental states, we graded day 3 embryos as "good" or "poor" embryos. On day 3 we defined "good quality" embryos as Grade 1 and Grade 2, with at least eight blastomeres and less than 20% cytoplasmic fragmentation. Embryos with unequal blastomere size and 20–50% fragmentation (Grades 3 and 4) were classified as "poor-quality" embryos.

Data analysis

Data were expressed as mean ± standard error (SE), and statistical analysis was carried out using the Mann-Whitney test (*U*-test). All analyses were performed using the Statistical Package for the Social Sciences (version 14.0; SPSS Inc, Chicago, IL). A confidence level of $P<0.05$ was the limit for statistical significance.

Results

Gene expression profiles in older (Cy3-labeled) and younger (Cy5-labeled) age groups were visualized with a scatterplot of Cy5 versus Cy3 intensity (Fig. 2). Analysis was conducted of 1879 genes, excluding genes that were absent, not found, or that had bad features. Most genes exhibited similar expression patterns between the two sample groups, but genes from certain functional groups were specific to either up- or down-regulation. There were 35 genes up-regulated (Table 3) and

Fig. 1 Amplification curves of the 11 studied genes in cumulus cells

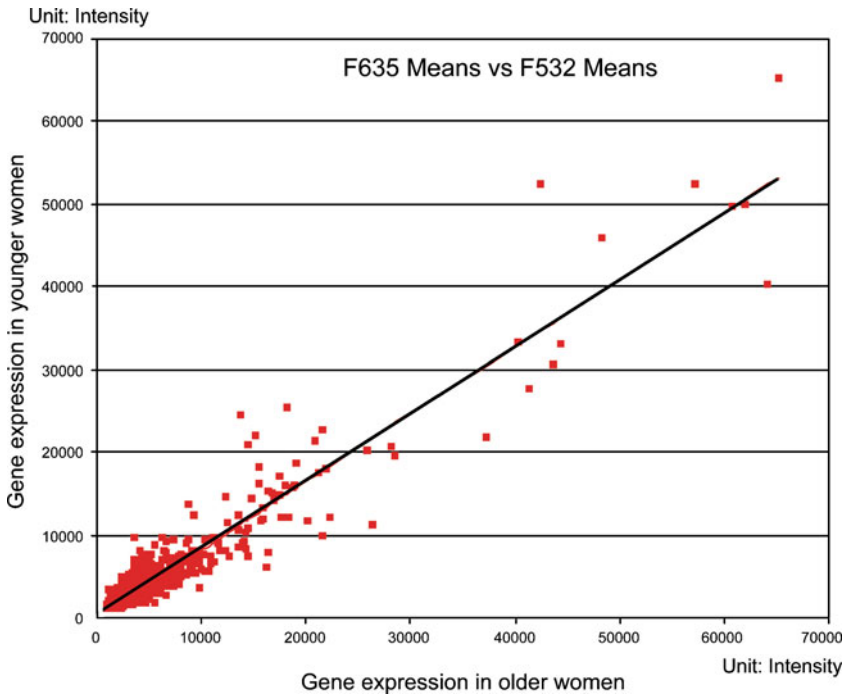

Fig. 2 Scatter plot representation of gene expression changes in older versus younger age group women. Cy3 intensity (samples from older women) is shown on the horizontal axis, and Cy5 intensity (samples from younger women) is represented on the vertical axis

Table 3 Thirty-five up-regulated genes in cumulus cell samples from older women, as determined by microarray analysis

No	Name	Gene_Symbol	Ratio of mean
1	serine (or cysteine) proteinase inhibitor- clade A (alpha-1 antiproteinase- antitrypsin)- member 3	SERPINA3	4.199
2	immediate early response 3	IER3	3.359
3	Simlarte a-actinin cytoskeletal isoform		2.916
4	hypothetical protein BC013995	LOC91663	2.748
5	Homo sapiens mRNA; cDNA DKFZp564K133 (from clone DKFZp564K133)		2.742
6	peroxiredoxin 2	PRDX2	2.699
7	eukaryotic translation initiation factor 5A	EIF5A	2.574
8	tubulin- beta- 5	TUBB5	2.499
9	ribosomal protein S28	RPS28	2.434
10	hydroxy-delta-5-steroid dehydrogenase- 3 beta- and steroid delta-isomerase 1	HSD3B1	2.320
11	cullin 1	CUL1	2.317
12	peptidylprolyl isomerase F (cyclophilin F)	PPIF	2.315
13	hypothetical protein BC013995	LOC91663	2.257
14	cysteine-rich- angiogenic inducer- 61	CYR61	2.253
15	cysteine-rich- angiogenic inducer- 61	CYR61	2.248
16	hypothetical protein BC013995	LOC91663	2.246
17	transforming growth factor- beta 1 (Camurati-Engelmann disease)	TGFB1	2.231
18	cytochrome P450- family 19- subfamily A- polypeptide 1	CYP19A1	2.215
19	major histocompatibility complex- class I- A	HLA-A	2.177
20	FBJ murine osteosarcoma viral oncogene homolog B	FOSB	2.123
21	interferon induced transmembrane protein 3 (1-8U)	IFITM3	2.100
22	metallothionein 1E (functional)	MT1E	2.099
23	Human HepG2 3′ region cDNA- clone hmd1f06.		2.090
24	hypothetical protein MGC4368	MGC 4368	2.088
25	non-metastatic cells 1- protein (NM23A) expressed in	NME1	2.072
26	creatine kinase- brain	CKB	2.056
27	cyclin-dependent kinase 4	CDK4	2.055
28	Homo sapiens mRNA; cDNA DKFZp666J2410 (from clone DKFZp666J2410)		2.049
29	peptidylprolyl isomerase B (cyclophilin B) poly(rC) binding protein 1	PCBP1	2.040
30	protein translocation complex beta	SEC61B	2.037
	cytochrome c oxidase subunit VIII	COX8	2.037
31	malate dehydrogenase 2- NAD (mitochondrial)	MDH2	2.033
32	stress-induced-phosphoprotein 1 (Hsp70/Hsp90-organizing protein)	STIP1	2.032
33	DKFZP586A0522 protein	DKFZP586A0522	2.028
34	ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	UCHL1	2.027
35	procollagen-proline- 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase)- beta polypeptide (protein disulfide isomerase; thyroid hormone binding protein p55)	P4HB	2.020

32 genes down-regulated in the older age group women. We identified genes with intensity changes greater than two-fold for further study; as a result, 11 genes were selected and grouped according to their function in cell cycle, apoptosis, and metabolism pathways (Table 1).

Total oocyte number and embryo number

Cumulus-oocyte complexes were collected from 38 women (see Materials and methods). The patients were divided into two groups according to age: ≤28 years old (younger age group, 12 patients) and ≥38 years old (older age group, 26 patients). The mean age, oocyte number, day 3 embryo number, and day 3 good-quality embryo number from these two groups are listed in Table 4. Younger patients had an average of 4.3±2.6 good-quality embryos on day 3, compared to 2.1±2.1 good-quality embryos in older patients.

Gene expression

According to the developmental states, we graded day 3 embryos as "good" or "poor" in accordance with previous

Table 4 Patient information and oocyte/embryo characteristics

Age group	Patients (no.)	Age (yr)	Retrieved oocytes (no.)	Day 3 embryos (no.)	Day 3 good-quality embryos (no.)	Pregnancy rate
≤28 years	12	*24.42±0.66	15.67±1.3	8.58±1.17	4.3±2.6	66.7%
≥38 years	26	40.08±0.26	7.92±0.89	3.96±0.64	2.1±2.1	34.6%

*Mean ± SE

methods (see Materials and methods) [17]. Good-quality embryos included grade 1, grade 2, and morula embryos, and the other grades of embryo were defined as poor-quality embryos. We did not directly assay gene expression levels in the embryo; we measured gene expression levels in cumulus cells retrieved from the corresponding oocyte that developed into the embryo. The expression levels of 11 genes, differentially expressed in cumulus cells of younger and older women, are shown in Fig. 3. Both *CKB* and *PRDX2* were significantly up-regulated in the older age group; there were no significant differences in the expression levels of the other 10 genes.

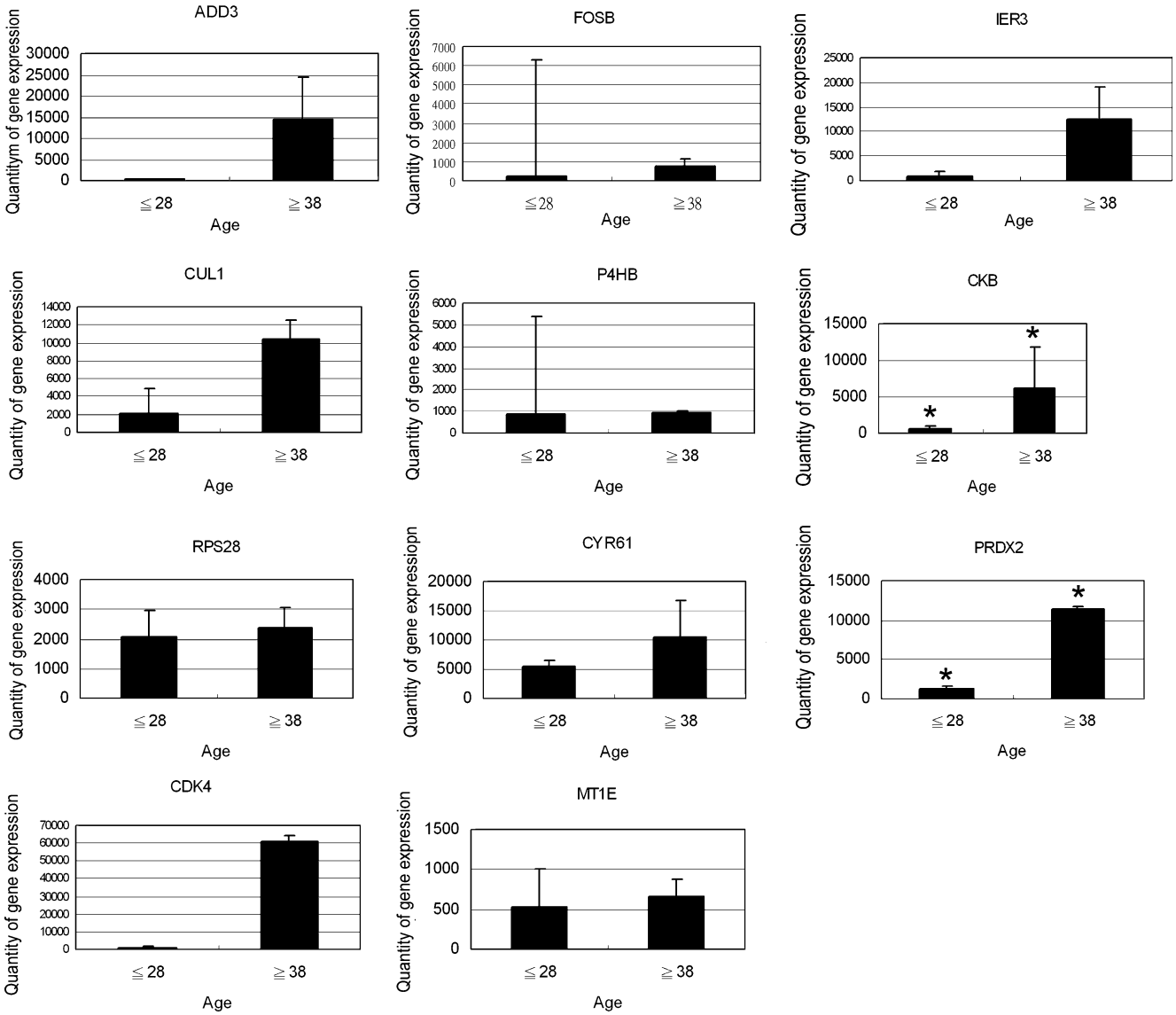

Fig. 3 Gene expression levels in cumulus cells from young (≦28 years) and advanced-age women (≧38 years). Data shown are (the quantities of gene expression/the quantity of *β-actin* expression) × 10000. *Significant differences between age groups ($P<0.05$, Mann-Whitney *U*-test)

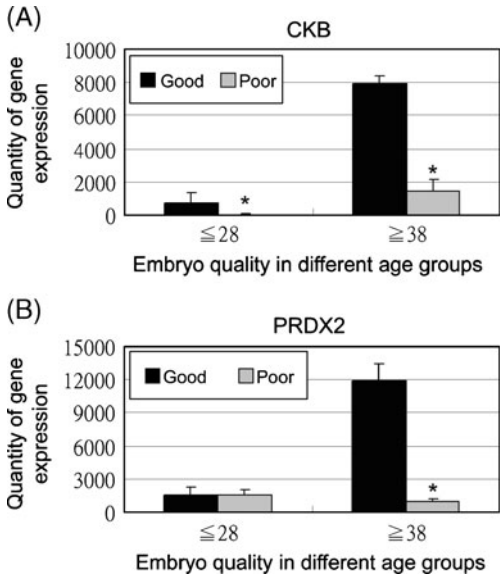

Fig. 4 *CKB* **a** and *PRDX2* **b** expression levels in cumulus cells compared with embryo development on day 3. Embryos were graded as described in Materials and methods. Results are divided into good-quality (black) and poor-quality (gray) embryos on day 3. *Significant difference between good- and poor-quality embryos in the same age group. ($P<0.05$, Mann-Whitney *U*-test)

Expression of *CKB* and *PRDX2* was significantly different between good-quality and poor-quality embryos. We also analyzed cumulus cell gene expression levels with reference to the grade of the associated embryo within each age group. *CKB* was expressed at a higher level by cumulus cells associated with good-quality embryos in both younger and older women. In addition, *PRDX2* was expressed at a higher level by cumulus cells associated with the good quality embryos in both younger and older women, but there was no significant difference. A higher level in *PRDX2* gene expression of cumulus cell was evident for samples acquired from older age group women than younger women for the gene (Fig. 4).

Discussion

The role of the surrounding cumulus cells in maturation, ovulation, and fertilization of oocytes has been extensively studied [4, 6], yet little is known about their role in oocyte aging. In order to potentially elucidate the interplay among cumulus cells and embryogenesis and oocyte aging, we have identified associations between the gene expression of selected cumulus cell genes, embryo quality, and the subject's age. Sperm quality and efficiency, super-ovulation, and gene expression may be influenced by environmental factors and/or ancillary genes that may have not been described or fully controlled in this study. We also have not accounted for the potential influence of secreted hormones.

Cumulus cell gene expression analysis may be useful in oocyte or embryo selection. Human cumulus and granulosa cells have been studied to identify biomarkers for oocyte quality and competence [18, 19], or for early embryo development [3, 20] or embryo quality and pregnancy outcome [21]. We randomly collected a subset of the cumulus cells from the subjects in this study, and some transferred embryos did not have their corresponding cumulus cells analyzed. Therefore, the pregnancy rates in this study (Table 4) cannot be compared directly to gene expression.

Previously, the expression of several genes in cumulus cells, particularly pentraxin-3, was indicative of oocyte and embryo quality [18]. In addition, the expression of cyclo-oxygenase 2 (COX2), gremlin (GREM), and hyaluronic acid synthase 2 (HAS2) were also positively correlation with embryo quality [20]. Oocyte factors such as growth and differentiation factor-9 (GDF-9) were necessary for cumulus expansion [3, 22], and the following cumulus cell-expressed genes were likely indicative of hypoxic conditions or delayed oocyte maturation: *CCND2, CXCR4, GPX3, CTNND1, DHCR7, DVL3, HSPB1*, and *TRIM28*. In the present study, we did not identify the same candidate genes as previous studies on embryo viability [3] or competent oocytes [19]. *CKB* catalyzes the reversible phosphorylation of creatine for cellular maintenance of the energy reservoir and transport. Cells that require high energy, such as spermatozoa, possess high CKB activity [23], and *CKB* expression was shown to be significantly altered in age-related-macular degeneration retinal tissue [24]. *CKB* expression was up-regulated in hormonally responsive tumors, including breast, prostate, and ovarian epithelial cancers [25, 26]. There may be a high level of estrogen response in the older age group following IVF treatment; *CKB* transcription and translation has been shown to be rapidly and specifically induced by estrogen [27, 28]. CKB was observed in the brain and uterus, and in the uterus its synthesis was increased by estrogen [29]. We observed a gnificant relationship between *CKB* expression and the age of the tested cumulus cells (Figs. 3 and 4). Follicular levels of estrogen tend be higher in older patients [30], and our older patients required higher dosages of exogenous gonadotropin for ovulation induction in IVF treatment cycles than the younger women. It is therefore likely that higher expression of *CKB* resulted from aging, perhaps mediated through estrogen responses.

CKB expression levels in cumulus cells were higher in samples acquired from older women, independent of whether these cumulus cells were associated with oocytes

that developed into good-quality or poor-quality embryos (Fig. 4). *CKB* activity occurs in growing mouse oocytes and in preimplantation embryos [31], and higher creatine kinase activities were associated with lower production of transferable embryos in dairy cattle [32]. Oxidative damage to the structure of oocytes and granulose cells, such as ruptured mitochondrial membranes and dilated smooth endoplasmic reticulum, was described in primordial follicles in a cohort of women of advanced age [33]. Increased CKB and superoxide dismutase (SOD) activities were observed in the presence of oxidative damage in spermatozoa, and SOD activities was highly correlated with creatine kinase in human sperm suspensions [34]. The female reproductive system may employ a similar mechanism to protect oocyte maturation and embryogenesis from oxygen damage. If the CKB genes are necessary to process oxidative stress or damage in cumulus cells, then older women would likely require higher levels of CKB to repair the oxidative damage. The higher CKB levels within each age group may be associated with a better capability to defend against oxidative stress, resulting in good-quality embryos.

The *PRDX2* gene encodes a member of the peroxiredoxin family of antioxidant enzymes that reduces hydrogen peroxide (H_2O_2) and alkyl hydroperoxides [35]. PRDX2 modulates intracellular H_2O_2 production and H_2O_2 - mediated apoptosis [36]. H_2O_2 production can therefore reduce the specific carbonyl level of PRDX2, suggesting that more active PRDX2 may be available in cumulus cells to strengthen the antioxidant system. Recent studies have demonstrated that the *PRDX* family of genes has varying patterns of gene expression in bovine oocytes and embryos [37]. Oxidative stress plays a role in multiple physiological processes, from oocyte maturation to fertilization and embryo development [38]; human granulosa cells and luteal cells respond to H_2O_2 by abolishing gonadotropin action and inhibiting progesterone [39]. Since PRDX2 modulates intracellular H_2O_2 production, it is possible that PRDX2 is responsible for elimination of H_2O_2 production in the follicle. The role of PRDX2 as an antioxidant may improve the biological function of cumulus cells and oocytes in older women. Cumulus cell gene expression was somewhat related to the age of the subject and the quality of the associated embryos. Since the functions of the *CKB* and *PRDX2* genes have not been fully elucidated, particularly regarding oogenesis and embryo development, it is difficult to interpret the gene expression patterns without further investigation.

Many studies exist regarding the relationship between embryo morphology and cumulus cell gene expression. A previous study identified *CCND2*, *CXCR4*, *GPX3*, *CTNND1*, *DHCR7*, *DVL3*, *HSPB1*, and *TRIM28* as differentially expressed genes, possibly reflecting hypoxic conditions or delayed oocyte maturation in non-early cleavage samples [40]. Three other genes expressed in cumulus cells were significantly associated with embryo quality, low fragmentation, or with seven cells [41]. These perspectives on new molecular embryo or oocyte selection parameters may be useful in countries where the selection has to be made at the oocyte stage before fertilization, instead of at the embryonic stage. The identification of biomarkers for noninvasive assessment of oocytes and embryos may also be aided by these findings.

As many failures in assisted reproduction may be related to oocyte aging, we measured gene expression levels in cumulus cells, anticipating that the cumulus cells may reflect the developmental potential of the oocyte. A study of nuclear transfer procedures revealed that aged oocytes showed low developmental potential as indicated by significantly lower fusion rates, cleavage rates, and subsequent development [42]. Indeed, it is well established that oocyte quality determines the embryo's post-fertilization development potential [43].

CKB and *PRDX2* expression in cumulus cells of older women was lower, and oocytes developed into poor-quality embryos on day 3 (Fig. 4). In good-quality embryos acquired from members of the same age group, *CKB* levels were higher in cumulus cells from older women than from younger women. Perhaps decreased *PRDX2* expression is associated with a decrease in antioxidant processes, thereby promoting apoptosis. The incidence of cumulus cell apoptosis has been used to predict the oocyte quality outcome of IVF-ET, as well as age-related declines in fertility [44]. These differences may affect oogenesis, which is responsible for the capacity to undergo normal fertilization and subsequent embryonic development, leading to the poor quality of the developing embryos.

In conclusion, we have observed variable expression of certain cumulus cell genes that, upon further study, may elucidate issues of human embryo development, possibly leading to the identification of markers of poor embryo development and potential therapeutic targets for improving embryo development, particularly in older women. *CKB* and *PRDX2*, which were expressed at higher levels in the cumulus cells of older women, may be candidates for evaluating embryo quality in the aging reproductive system.

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References

1. Borini A, Lagalla C, Cattoli M, Sereni E, Sciajno R, Flamigni C, et al. Predictive factors for embryo implantation potential. Reprod Biomed Online. 2005;10:653–68.
2. Gerris JM. Single embryo transfer and IVF/ICSI outcome: a balanced appraisal. Hum Reprod Update. 2005;11:105–21.
3. van Montfoort AP, Geraedts JP, Dumoulin JC, Stassen AP, Evers JL, Ayoubi TA. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. Mol Hhum Reprod. 2008;14:157–68.
4. Eppig JJ. The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Dev Biol. 1982;89:268–72.
5. Eppig JJ. Intercommunication between mammalian oocytes and companion somatic cells. Bioessays. 1991;13:569–74.
6. Tanghe S, Van Soom A, Nauwynck H, Coryn M, de Kruif A. Functions of the cumulus oophorus during oocyte maturation, ovulation, and fertilization. Mol Reprod Dev. 2002;61:414–24.
7. Thomas FH, Vanderhyden BC. Oocyte-granulosa cell interactions during mouse follicular development: Regulation of kit ligand expression and its role in oocyte growth. Reprod Biol Endocrinol. 2006;4:19–26.
8. Cecconi S, Tatone C, Buccione R, Mangia F, Colonna R. Granulosa cell-oocyte interactions: the phosphorylation of specific proteins in mouse oocytes at the germinal vesicle stage is dependent upon the differentiative state of companion somatic cells. J Exp Zool. 1991;258:249–54.
9. Eppig JJ, Chesnel F, Hirao Y, O'Brien MJ, Pendola FL, Watanabe S, et al. Oocyte control of granulosa cell development: how and why. Hum Reprod. 1997;12:127–32.
10. Chang H, Brown CW, Matzuk MM. Genetic analysis of the mammalian transforming growth factor-beta superfamily. Endocr Rev. 2002;23:787–823.
11. Vanderhyden BC, Macdonald EA, Nagyova E, Dhawan A. Evaluation of members of the TGF beta superfamily as candidates for the oocyte factors that control mouse cumulus expansion and steroidogenesis. Reprod. 2003;61:55–70.
12. Faddy MJ, Gosden RG, Gougeon A, Richardson SJ, Nelson JF. Accelerated disappearance of ovarian follicles in mid-life: implications for forecasting menopause. Hum Reprod. 1992;7:1342–6.
13. te Velde E, Pearson P. The variability of female reproductive aging. Hum Reprod Update. 2002;8:141–54.
14. Janny L, Menezo YJ. Maternal age effect on early human embryonic development and blastocyst formation. Mol Reprod Devel. 1996;45:31–7.
15. Navot D, Bergh PA, Williams MA, Garrisi GJ, Guzman I, Sandler B, et al. Poor oocyte quality rather than implantation failure as a cause of age-related decline in female fertility. Lancet. 1991;337:1375–7.
16. Bassil S, Wyns C, Toussaint-Demylle D, Abdelnour W, Donnez J. Predictive factors of multiple pregnancy in in vitro fertilization. J Reprode Med. 1997;42:761–6.
17. Lin DP, Huang CC, Wu HM, Cheng TC, Chen CI, Lee MS. Comparison of mitochondrial DNA contents in human embryos with good or poor morphology at the 8-cell stage. Fertil Steril. 2004;81:73–9.
18. Zhang X, Jafari N, Barnes RB, Confino E, Milad M, Kazer RR. Studies of gene expression in human cumulus cells indicate pentraxin 3 as a possible marker for oocyte quality. Fertil Steril. 2005;83:1169–79.
19. Hamel M, Dufort I, Robert C, Gravel C, Leveille MC, Leader A, et al. Identification of differentially expressed markers in human follicular cells associated with competent oocytes. Hum Reprod. 2008;23:1118–27.
20. McKenzie LJ, Pangas SA, Carson SA, Kovanci E, Cisneros P, Buster JE, et al. Human cumulus granulosa cell gene expression: a predictor of fertilization and embryo selection in women undergoing IVF. Hum Reprod. 2004;19:2869–74.
21. Assou S, Haouzi D, Mahmoud K, Aouacheria A, Guillemin Y, Pantesco V, et al. A non-invasive test for assessing embryo potential by gene expression profiles of human cumulus cells: a proof of concept study. Mol Hum Reprod. 2008;14:711–9.
22. Sutton ML, Gilchrist RB, Thompson JG. Effects of in-vivo and in-vitro environments on the metabolism of the cumulus-oocyte complex and its influence on oocyte developmental capacity. Hum Reprod Update. 2003;9:35–48.
23. Rolf C, Behre HM, Cooper TG, Koppers B, Nieschlag E. Creatine kinase activity in human spermatozoa and seminal plasma lacks predictive value for male fertility in in vitro fertilization. Fertil Steril. 1998;69:727–34.
24. Yoshida S, Yashar BM, Hiriyanna S, Swaroop A. Microarray analysis of gene expression in the aging human retina. Invest Ophthalmol Vis Sci. 2002;4:2554–60.
25. Zarghami N, Yu H, Diamandis EP, Sutherland DJ. Quantification of creatine kinase BB isoenzyme in tumor cytosols and serum with an ultrasensitive time-resolved immunofluorometric technique. Clin Biochem. 1995;28:243–53.
26. Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC. Clinical applications of microarray technology: creatine kinase B is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. Gyneol Oncol. 2005;96:77–83.
27. Wu-Peng XS, Pugliese TE, Dickerman HW, Pentecost BT. Delineation of sites mediating estrogen regulation of the rat creatine kinase B gene. Mol Endocri. 1992;6:231–40.
28. Zarghami N, Giai M, Yu H, Roagna R, Ponzone R, Katsaros D, et al. Creatine kinase BB isoenzyme levels in tumour cytosols and survival of breast cancer patients. Br J Cancer. 1996;73:386–90.
29. Bergen HT, Pentecost BT, Dickerman HW, Pfaff DW. In situ hybridization for creatine kinase-B messenger RNA in rat uterus and brain. Mol Cellular Endocrinol. 1993;92:111–9.
30. Welt CK, Jimenez Y, Sluss PM, Smith PC, Hall JE. Control of estradiol secretion in reproductive ageing. Hum Reprod. 2006;21:2189–93.
31. Iyengar MR, Iyengar CW, Chen HY, Brinster RL, Bornslaeger E, Schultz RM. Expression of creatine kinase isoenzyme during oogenesis and embryogenesis in the mouse. Dev Biol. 1983;96:263–8.
32. Chorfi Y, Lanevschi A, Dupras R, Girard V, Tremblay A. Serum biochemical parameters and embryo production during superovulatory treatment in dairy cattle. Res Vet Sci. 2007;83:318–21.
33. de Bruin JP, Dorland M, Spek ER, Posthuma G, van Haaften M, Looman CW, et al. Age-related changes in the ultrastructure of the resting follicle pool in human ovaries. Biol Reprod. 2004;70:419–24.
34. Aitken RJ, Buckingham DW, Carreras A, Irvine DS. Superoxide dismutase in human sperm suspensions: relationship with cellular composition, oxidative stress, and sperm function. Free Radic Biol Med. 1996;21:495–504.
35. Wood ZA, Schröder E, Robin HJ, Poole LB. Structure, mechanism and regulation of peroxiredoxins. Trends Biochem Sci. 2003;28:32–40.
36. Kim H, Lee TH, Park ES, Suh JM, Park SJ, Chung HK, et al. Role of peroxiredoxins in regulating intracellular hydrogen peroxide and hydrogen peroxide induced apoptosis in thyroid cells. J Biol Chem. 2000;275:18266–70.
37. Leyens G, Knoops B, Donnay I. Expression of peroxiredoxins in bovine oocytes and embryos produced in vitro. Mol Reprod Dev. 2004;69:243–51.

38. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprode Biol Endocrnol. 2005;14:28–49.
39. Vega M, Carrasco I, Castillo T, Troncoso JL, Videla LA, Devoto L. Functional luteolysis in response to hydrogen peroxide in human luteal cells. J Endocrinol. 1995;147:177–82.
40. van Montfoort APA, Geraedts JPM, Dumoulin JCM, Stassen APM, Johannes LH, Evers JLH, et al. Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis. MHR-Basic Science of Reproductive Medicine. 2008;14:157–68.
41. Adriaenssens T, Wathlet S, Segers I, Verheyen G, De Vos A, Van der Elst J, et al. Cumulus cell gene expression is associated with oocyte developmental quality and influenced by patient and treatment characteristics. Hum Reprod. 2010; 1:1–12.
42. Hall VJ, Compton D, Stojkovic P, Nesbitt M, Herbert M, Murdoch A, et al. Developmental competence of human in vitro aged oocytes as host cells for nuclear transfer. Hum Reprod. 2007;22:52–62.
43. Wang Q, Sun QY. Evaluation of oocyte quality: morphological, cellular and molecular predictors. Reprod Fertil Dev. 2007;19:1–12.
44. Lee KS, Joo BS, Na YJ, Yoon MS, Choi OH, Kim WW. Cumulus cells apoptosis as an indicator to predict the quality of oocytes and the outcome of IVF-ET. J Assist Reprod Genet. 2001;18:490–8.