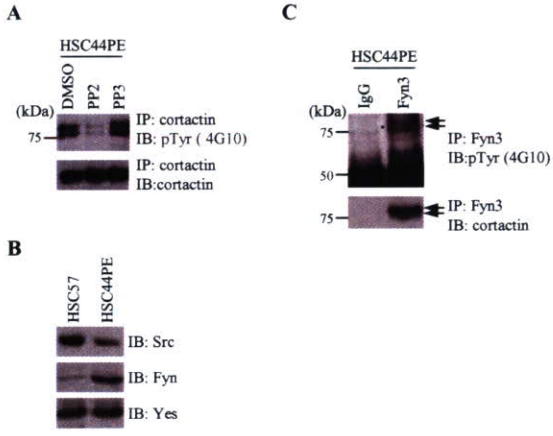
Supplementary Figure

Involvement of SFKs in gastric cancer cells HSC44PE. (A) Src family inhibitor PP2 impaired tyrosine phosphorylation of cortactin in HSC44PE gastric cancer cells. HSC44PE cells were treated with 10μM PP2 or PP3 for 3 hours prior to cell lyses. The whole-cell lysates were immunoprecipitated by anti-cortactin antibody, and immunoblotted by anti-phosphotyrosine antibody 4G10. **(B)** Expression of three major Src family members (Src, Fyn and Yes) in HSC57 and HSC44PE cells. Total 20 μg cell lysates were subjected to immunoblotting analysis by anti-Src, anti-Fyn3 and anti-Yes antibodies. (C) Lysates of HSC44PE cells were immunoprecipitated by anti-Fyn3 antibody or rabbit Ig-G (negative control), and subjected to immunoblotting by anti-cortactin antibody. The position of cortactin protein is indicated by the arrow.

Supplementary Figure



Glutathione S-transferase theta 1 expressed in granulosa cells as a biomarker for oocyte quality in age-related infertility

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Objective: The goal of this study was to identify a reliable biomarker for age-related infertility.
Design: Laboratory study.
Setting: ART laboratory.
Patient(s): Patients undergoing intracytoplasmic sperm injection or IVF cycles.
Intervention(s): Expression of Glutathione S-transferase (GST) mRNA and protein in mural and cumulus granulosa cells obtained from infertile patients were examined by reverse transcriptase-polymerase chain reaction and immunofluorescence.
Main Outcome Measure(s): Correlation between the expression of GST theta 1 (GSTT1) in granulosa cells and oocyte quality was a main outcome measure.
Result(s): Expression of GSTT1 in granulosa cells from male factor patients was positively correlated with age and negatively with cumulus-oocyte complex maturity. When samples with high and low GSTT1 in granulosa cells were extracted from the other infertility factors, cumulus-oocyte complex maturity in the high GSTT1 group was significantly lower than that in the low GSTT1 group (high: 27.2% vs. low: 51.3%). The developmental capacity of oocytes in the high GSTT1 group was likely to be lower (high: 26.4% vs. low: 43.9%). Up-regulation of GSTT1 during aging may be promoted by FSH and H_2O_2, determined by an in vitro model.
Conclusion(s): GSTT1 is a good indicator for age-related infertility. (Fertil Steril® 2007;■:■–■. ©2007 by American Society for Reproductive Medicine.)

Key Words: Glutathione S-transferase theta 1 (GSTT1), aging, granulosa cell, biomarker, oocyte quality

Maternal age is a risk factor for infertility. The decline in fecundity becomes clinically evident when women reach their mid-30s (1). Despite the disadvantages, women in Western industrialized nations now tend to delay the birth of their first child until a later age than before. This tendency leads to declining birth rates, resulting in aging populations, which is a serious social issue. Although the exact mechanism by which aging causes female reproductive disorders is unclear, age-related changes in the ovary—including hormonal imbalance (2), decrease of the ovarian follicle pool (3), increase of oocyte aneuploidy (4), and mitochondrial dysfunction in oocytes (5)—account for the loss of reproductive function.

Oxidative stress is a major source of aging; it damages genomic and mitochondrial DNA, causing tumors and/or apoptosis in many cell types. Oocytes and somatic cells stored in ovaries are thought to be exposed to reactive oxygen species (ROS) during both ovulation and aging (6). In mice, repeated ovarian stimulation with gonadotropins increased the

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incidence of oxidative DNA damage and mitochondrial DNA mutation (7). In humans, accumulation of 8-hydroxy-2'-deoxyguanine, a byproduct of oxidative stress-induced digestion of DNA, in mural and cumulus granulosa cells from infertile patients correlated negatively with the oocyte quality (8). Moreover, the amount of ROS in human follicular fluid was negatively correlated with oocyte development potential (9). The apoptotic status of rat follicular cells was also in parallel with ROS production (10). In agreement with this report, apoptosis possibly induced by ROS in granulosa cells from infertile patients was closely related to the oocyte quality (11, 12). Thus, repeated exposure of oocytes and granulosa cells to oxidative stress must be associated with reproductive failure.

Organisms have many adaptive devices to oxidative stress and genotoxins. Glutathione S-transferases (GSTs) are well known to detoxify the metabolites of genotoxic molecules to more water-soluble and readily excretable forms. In addition, they are known to protect cells from ROS-induced membrane lipid peroxidation (13). Because of their roles in self-defense, mutations of GSTs are often linked to certain diseases. Several studies have indicated that GSTs may play a role in predisposition to cancer, with the GSTM1 and GSTT1 null phenotypes (14–16). A number of common polymorphisms affect enzyme activity; these include gene deletions in the GSTM1 and GSTT1 genes, which result in lack of the corresponding enzyme activity (17). The mutated products modulate chemical binding to DNA, and are associated with myocardial infarction as well as the tobacco-related cancers in smokers (18, 19). In addition, polymorphisms of GSTM1 and GSTT1 may increase the risk of recurrent pregnancy loss (20), and susceptibility to polycystic ovaries (21).

Because of the limited availability of molecular information and biomarkers for age-related infertility, effective diagnosis and therapy have not yet been established. The aim of our present study, therefore, was to explore the possibility that the expression of GSTs in granulosa cells obtained from infertility patients is associated with age-related changes of fecundity. We screened for the expression of GSTs in young or older (stressed) granulosa cells by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, to select GSTs as potential biomarkers for age-related infertility; we then compared the expression levels of each potential molecule in various patients with oocyte quality. Finally, we examined the cause of aberrant expression of GSTs in in vitro model systems.

MATERIALS AND METHODS

Reagents

Hoechst 33342, human FSH, human LH, and hCG were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against GSTT1 and BAX were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Donkey anti-rabbit IgG-Cy3 was purchased from Chemicon International Co. Ltd. (Temecula, CA).

Patients and Samples

Mural granulosa cells and cumulus granulosa cells were obtained from infertile patients undergoing IVF/intracytoplasmic sperm injection cycles between September 2004 and June 2007 at the National Center for Child Health and Development, Japan. One hundred seventy-six infertile patients (male factor: 50; tubal factor: 32; unknown factor: 94) participated in the study. The institutional review board approved the experiments on these samples, and individual patients provided prior informed consent.

Mural granulosa cells were isolated from follicular aspirates and washed three to four times in phosphate-buffered saline (PBS) containing 1 mg/mL bovine serum albumin (BSA) (PBS + BSA). In addition, cumulus-oocyte complexes (COCs) were isolated and washed twice in IVF medium (human tubal fluid medium purchased from Irvine Scientific Co., Santa Ana, CA). Cumulus cells were then detached from the COCs physically using 27-G fine needles in IVF medium and washed three times in PBS + BSA. Just before detachment of the cumulus cells, the maturity of the COCs was evaluated based on the following morphologic criteria: [1] complete expansion of cumulus with visible halo (mature), [2] incomplete expansion of cumulus without halo (immature), and [3] incomplete or complete expansion and dissociation of cumulus with dark spots (dysmature).

A portion of the mural and cumulus granulosa cells was transferred into 1.5-mL microtubes, centrifuged for 10 minutes at 15,000 rpm at 4°C to remove excess buffer, and stored at −80°C until use. The remaining samples were fixed in 4% formaldehyde in PBS + BSA for 30 minutes at room temperature, washed three times in PBS + BSA, put on glass slides, air-dried, and stored at 4°C until use.

Preparation of Mouse Ovaries

Experiments were approved by the Animal Ethics Committee at the National Center for Child Health and Development. ICR female mice (8 weeks old) were purchased from Sankyo Labo Service Co. Ltd (Tokyo, Japan). They were injected with 6 IU of pregnant mare serum gonodotropin (PMSG), and ovaries were collected at 72 hours post-injection to induce the atretic follicles. In addition, some mice were injected with 6 IU hCG following PMSG administration, and ovaries were collected at 8 hours post-hCG injection to obtain ovaries with preovulatory follicles. Those ovaries were then fixed in 4% paraformaldehyde in PBS overnight at 4°C. They were then sectioned serially by Cryostat (8 μm interval, Leica Microsystems Japan Co. Ltd, Tokyo, Japan), and subjected to immunofluorescence studies. The sections were kept at 4°C until use.

Cell Culture and Treatment

A human granulosa-like tumor cell line, KGN, was established previously (22). The cells were maintained in Dulbecco's minimum essential medium/Ham's F12 medium supplemented with 10% heat-inactivated fetal calf serum,

100 μg/mL penicillin, and 100 IU/mL streptomycin at 37°C in a CO_2 incubator. For the immunofluorescence study, the cells were seeded onto eight-well culture slides (BD Japan Co. Ltd, Tokyo, Japan) and stimulated with either gonadotropins (FSH, LH, hCG) or oxidative stress (H_2O_2, 10 μM) for 6 to 24 hours at the indicated concentration. The cells were incubated in serum-free culture medium for 2 hours before treatment with the various stimuli.

Semiquantitative RT-PCR Analysis

Total RNA was isolated from mural granulosa cells using ISOGEN RNA extraction reagent (Nippon Gene Co. Ltd., Tokyo, Japan). The first-strand cDNA of each sample was synthesized from 1 μg total RNA using SuperScript III reverse transcriptase (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. Expression of GSTs was then monitored by PCR amplification. The primers and cycles used for each gene are as follows; GSTA1 F: GATTTGTTTTCATTAGGATCTGA, R: CATGGAGAAGA TTGGAAATCTGAAT, 32 cycles; GSTA2 F: CAGCCACA AAGGTGACAGCA, R: TCAGGTTGAGTAAAGGGCAG, 32 cycles; GSTA3 F: GGTGGCCTTGAGAAGCTGAG, R: TGGTTTCTTTGAGTAAAGTCT, 35 cycles; GSTA4 F: GGCTATAATTAGATACTTTCCTGTG, R: GGGATATT ACTTAGTTTCACTGTGT, 28 cycles; GSTCD1 F: CTTTAT CATACAAGGAACACATGAT, R: ACGGAGTATCCACAT TCTTCT, 32 cycles; GSTCD2 F: CTTTGAAATAAACTTT CAAGACTCAG, R: AAGATACCAAAAGAATATGCTCA, 32 cycles; GSTK1 F: GTATCAGAATATCTGGAACATCAA C, R: CTTTAAGTCATTTGCCATGTATAGT, 28 cycles; GSTM1 F: AAGCTATGAGGAAAAGAAGTACAC, R: ATA GACGAGAAAATCTACAAAAGTG, 28 cycles; GSTM2 F: AAGGAGCAGATTCGCGAAGAC, R: GCCTGCAGGT ATTCTGGTTTC, 28 cycles; GSTM3 F: CCCCCTTATGTA GGGTATAAAG, R: TCATAAGAGGTATCCGTGAACT, 35 cycles; GSTM4 F: ATCTTACTCCTTCCAGCCAGT, R: ACATGATGCTGGTTGGTGTC, 32 cycles; GSTM5 F: GATTACTACAGAAAGGTGCTCTG, R: TATCTAGATC AGGGAAGTCTATCAG, 32 cycles; GSTO1 F: GATCTTAG AGTTGTTTTCTAAGGTG, R: TTCTTATTAGTCAGAAC CTCCTCTA, 28 cycles; GSTO2 F: ACCACTACCGGTTCC TCTTT, R: GTGATCCTACCCGCCAACTA, 42 cycles; GSTP F: ATCTCCCTCATCTACACCAACTAT, R: AGTCC AGCAGGTTGTAGTCAG, 28 cycles; GSTT1 F: GGATCTG ATTAAAGGTCAGCACTTA, R: ACCAGTAGTCAGGGA CCTTATATTT, 35 cycles; GSTT2 F: AACAAGGATTCA TTCTGTTACTTAC, R: TTACTGATTTGTAGGAGCTCTT TAT, 32 cycles; GSTZ1 F: ATATACTGTGTAGGAGACG AGGTG, R: CGTCACATAGATAACAGTATTCCT, 32 cycles; MGST1 F: ACCTGTAAAGAAAATCATACAACTC, R: CCTCCTACCTAAAATCTAAGAAAGT, 28 cycles; MGST2 F: GTGTGGAGTTTTATCCTATATTCAT, R: CAT TAAAGGGAAGAGAAAAAGTTAG, 28 cycles; MGST3 F: AAGAAGTACAAAGTGGAGTATCCTA, R: GTAATAG CCATAAGCATAAAGAACT, 28 cycles; G3PDH F: ACCAC AGTCCATGCCATCAC, R: TCCACCACCCTGTTGCTGT A, 28 cycles. The conditions for PCR amplification were: initial denaturation at 94°C for 5 minutes, followed by the indicated cycles of denaturation 94°C for 45 seconds, annealing at 57°C for 45 seconds, and elongation at 72°C for 1 minute, with a final extension at 72°C for 15 minute.

The PCR products were separated on a 4% agarose gel and stained with ethidium bromide. Digital photographs were taken on a transilluminator and saved as JPEG files. The averaged band intensity was measured using Adobe Photoshop Element 2 software and the background was subtracted. Semiquantitative data on the expression level of each gene were obtained from values that had been divided by those for G3PDH.

Immunofluorescence

Mural and cumulus granulosa cells prepared as described above were permeabilized with 0.1% Triton X-100 (TX100) in PBS for 30 minutes at room temperature and blocked with 100% Blockace (Snow Brand Milk Products Co., Tokyo, Japan) overnight at room temperature. Cryosections were also blocked with 100% Blockace. The samples were treated with the first antibody at 20 μg/mL for 2 hours at room temperature, washed three times with PBS, and visualized with anti-rabbit IgG–Cy3 diluted at 1:500 in PBS + 0.1% TX100 for 2 hours at room temperature. To count the number of cells, Hoechst 33342 was loaded at 10 μM in PBS + 0.1% TX100 during treatment with the secondary antibody. The samples were then washed three times with PBS, immersed in Vectashield (Vector Laboratories Inc., Burlingame, CA), and covered with coverslips. Microphotographs were taken under an epifluorescence microscope (IX-71; Olympus, Tokyo, Japan) equipped with a computational CCD camera (CoolSnap HQ; Nippon Roper Industries, Tokyo, Japan). Photographs taken from five different areas of each sample were analyzed with MetaMorph software (Molecular Devices Corp., Tokyo, Japan), and the averaged fluorescence intensity in each image was measured. The fluorescence intensity was normalized with the number of cells in the same image to obtain the indexed fluorescence intensity per cell.

Statistical Analysis

Normalized data on the expression of GSTT1 mRNA and protein were presented as means with SEM from three to five independent experiments. Correlation between GSTT1 mRNA expression and oocyte quality (cumulus maturity and developmental capacity) was estimated by Pearson's coefficients of contingence. Expression of GSTT1 or BAX protein in mural and cumulus granulosa cells from young patients was compared with that of older patients using Student's *t* test or modified *t* test (Welch's correction). The expression levels of GSTT1 protein in KGN cells with various treatments were analyzed with one-way analysis of variance followed with Student's *t* test. Differences were considered statistically significant when $P<.05$. All statistical analyses were performed using Microsoft Excel software.

RESULTS

Up-regulation of GSTT1 in Mural Granulosa Cells with Aging

In most cases of male factor infertility, female fecundity is considered normal. Accordingly, oocyte quality in the women with male factor must be affected by aging. We therefore tried to select a typical age sample from 50 male factor patients, considering the age of patients and the availability of granulosa cells for the subsequent experiments. The demographic data on the patients participating in this study are summarized in Table 1.

Unlike most GSTs, GSTT1 was found to be up-regulated in aged mural granulosa cells (Fig. 1A). To explore the possibility that GSTT1 might be useful as a biomarker of age-related infertility, the correlation between GSTT1 mRNA expression in granulosa cells from male factor patients and the age of the patients was analyzed (N = 43). As shown in Figure 1B, a positive correlation between GSTT1 level and age was observed ($r = 0.36$, $P<.05$). Immunofluorescence analysis of GSTT1 in mural granulosa cells revealed that the aged cells expressed GSTT1 about 10-fold more strongly than young cells (Fig. 1C; young: 25–34 years, N = 9; aged: 38–43 years, N = 12, $P<.01$). A similar tendency was observed in cumulus granulosa cells (Fig. 1D; young: 25–34 years, N = 7; aged: 37–42 years, N = 5), although the difference was not statistically significant. These results demonstrate that GSTT1 in granulosa cells was up-regulated by aging.

GSTT1 Predicts Oocyte Quality

Because GSTT1 was shown to be a marker for aging, we next analyzed the correlation between GSTT1 expression in male factor patients and oocyte quality (number of oocytes retrieved, COC maturity, fertilization rate, and developmental rate). A negative correlation between GSTT1 mRNA expression and COC maturity was observed (Fig. 2A; $r = -0.31$, $P<.05$). Immunofluorescence revealed that GSTT1 protein in cumulus cells from the immature and dysmature COCs was up-regulated significantly compared with that from the mature COCs (Fig. 2B; N = 5, mature vs. immature, $P<.05$; mature vs. dysmature, $P<.01$). These results demonstrate that GSTT1 was an indicator for the COC maturity.

Although the COC maturity is not related to the nuclear maturity of oocytes, it may represent the apoptotic status of cumulus cells. Therefore, expression of the BAX protein, an apoptosis marker, in cumulus cells was examined by immunofluorescence. BAX expression was greatest in the dysmature cumulus cells, modest in the immature cells, and least in the mature cells (Fig. 2C; N = 6, mature vs. dysmature: $P<.05$). Supportively, both GSTT1 and BAX were expressed very strongly in mouse atretic follicles, whereas the minimum fluorescence of those molecules was seen in granulosa cells in preovulatory follicles (Fig. 2D). These results suggest that GSTT1 was an indicator for the apoptosis of the granulosa cells.

The correlation between GSTT1 expression and oocyte capacity for fertilization and development could not be examined in the male factor patients because these are affected by sperm quality. We therefore tested the expression of GSTT1 mRNA in the mural granulosa cells from other infertile patients (tubal and unknown factors), and compared this with oocyte capacities. In these cases, GSTT1 expression showed no correlations with COC maturity (Fig. 3A) or oocyte capacity for fertilization (data not shown) or development, possibly because GSTT1 expression was affected not only by aging but also by various other factors. However, when patients with high (more than the G3PDH level) or low (<1/5 of the G3PDH level) expression of GSTT1 in granulosa cells were extracted, a negative correlation between GSTT1 expression and COC maturity was observed ($r = -0.35$, $P<.05$; data not shown). The average rate of COC maturity in the group with high GSTT1 was also significantly lower than that in the group with low GSTT1 (Fig. 3B; low: 51.3%, N = 25; high: 29.5%, N = 17, $P<.01$). Therefore, defining the threshold of GSTT1 expression could be effective for extraction of the aging factor in infertile patients. In the

TABLE 1

Demographic data of IVF/ICSI patients.

		Selected samples for GST screening[d]	
	All samples	Young	Aged
Age (years)	37.3 ± 0.4	28	36
Cases of each cause			
Male factors	50		
Tubal	32		
Unknown	94		
Day 3 FSH (mIU/mL)	5.3 ± 0.2	2.5	5.1
No. of hMG ampoules[a]	23.7 ± 0.6	17	28
No. oocytes retrieved	8.3 ± 0.4	10	13
% maturity of COC[b]	48.4 ± 2.3	40	46.2
% fertilization	57.2±2.3	10	15.4
% development[c]	32.5±2.3	0	0
% pregnancy	17.0	—	—

Note: COC = cumulus–oocyte complex. Values are means ± SEM. Data are obtained from 176 patients unless described.

[a] One hMG ampoule containts 75 IU.

[b] Percentage of mature COC evaluated by morphology.

[c] Percentage of oocytes developed to eight-cell stage or later at Day 3 post-IVF/intracytoplasmic sperm injection.

[d] Representative samples were selected from 50 male factor patients for GST expression profiling. The averaged age of 50 male factor patients was 35.2 ± 0.8 years old.

Ito. GSTT1 as a marker for age-related infertility. Fertil Steril 2007.

FIGURE 1

Up-regulation of GSTT1 in granulosa cells with aging. (**A**) Relative expression of GST isoform mRNA in representative mural granulosa cells from young (28 years) and older (36 years) male factor patients. The difference in the expression of each GST mRNA is shown as the mean log of fold increase with SEM from three independent experiments. (**B**) Correlation between GSTT1 mRNA expression in mural granulosa cells from male factor patients and the age of the patients (N = 43). Statistical analysis was conducted using Pearson's correlation contingence ($r = 0.36$, $P < .05$). (**C, D**) Immunostaining of GSTT1 protein (red) in mural (**C**) and cumulus (**D**) granulosa cells. *Left panels* show typical microphotographs from young (**C**: 25 years, **D**: 28 years) and older (**C**: 38 years, **D**: 38 years) patients. The cells were counterstained with Hoechst 33342 at 10 μM to visualize the nuclei (blue). *Right panels* show the mean fluorescence intensity of GSTT1 per cell with SEM from young (**C**: N = 9, 25–34 years, **D**: N = 7, 25–34 years) and older (**C**: N = 12, 38–43 years, **D**: N = 5, 37–42 years) patients. The data were analyzed by Student's or modified *t* test (**$P < .01$).

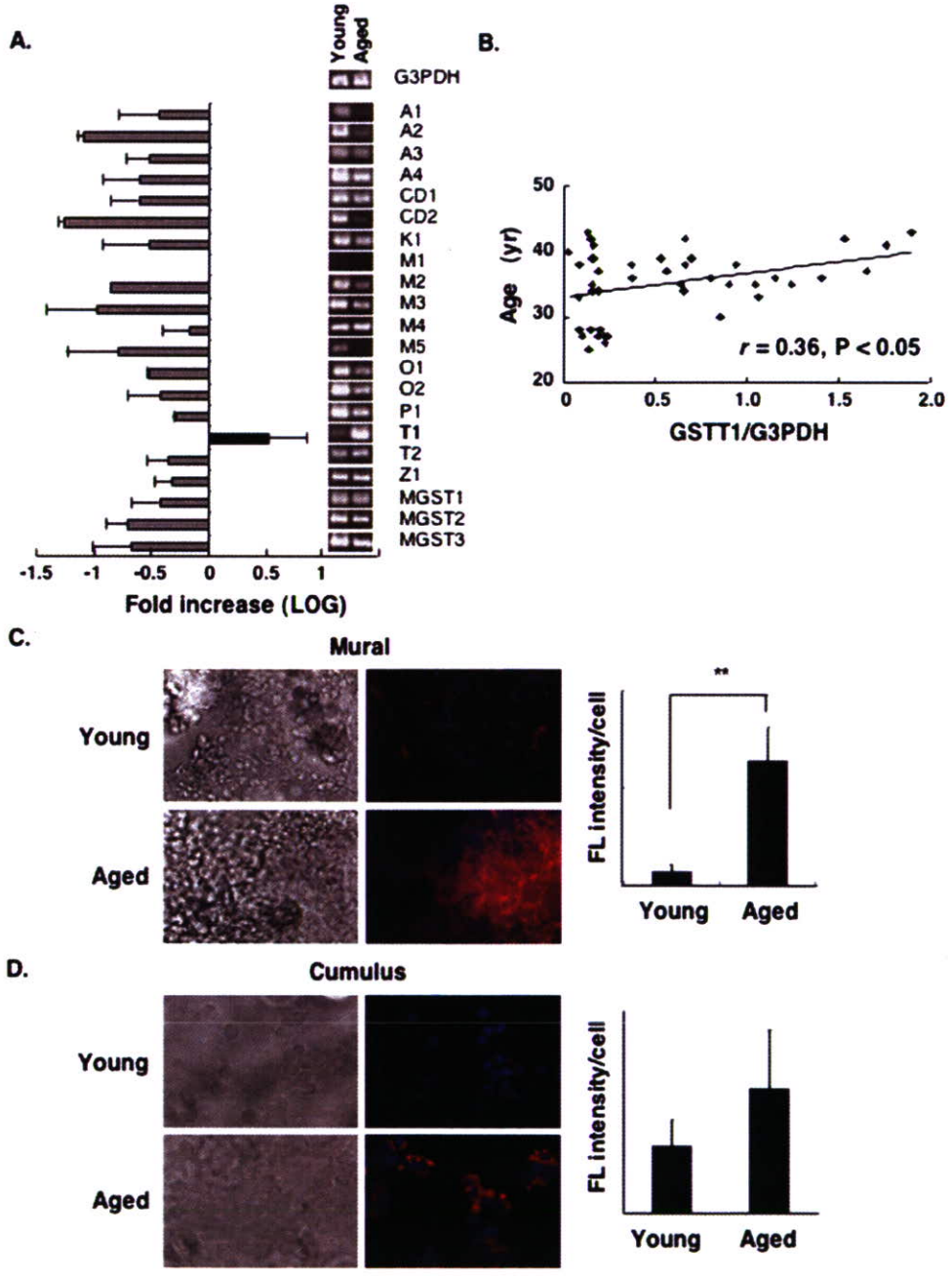

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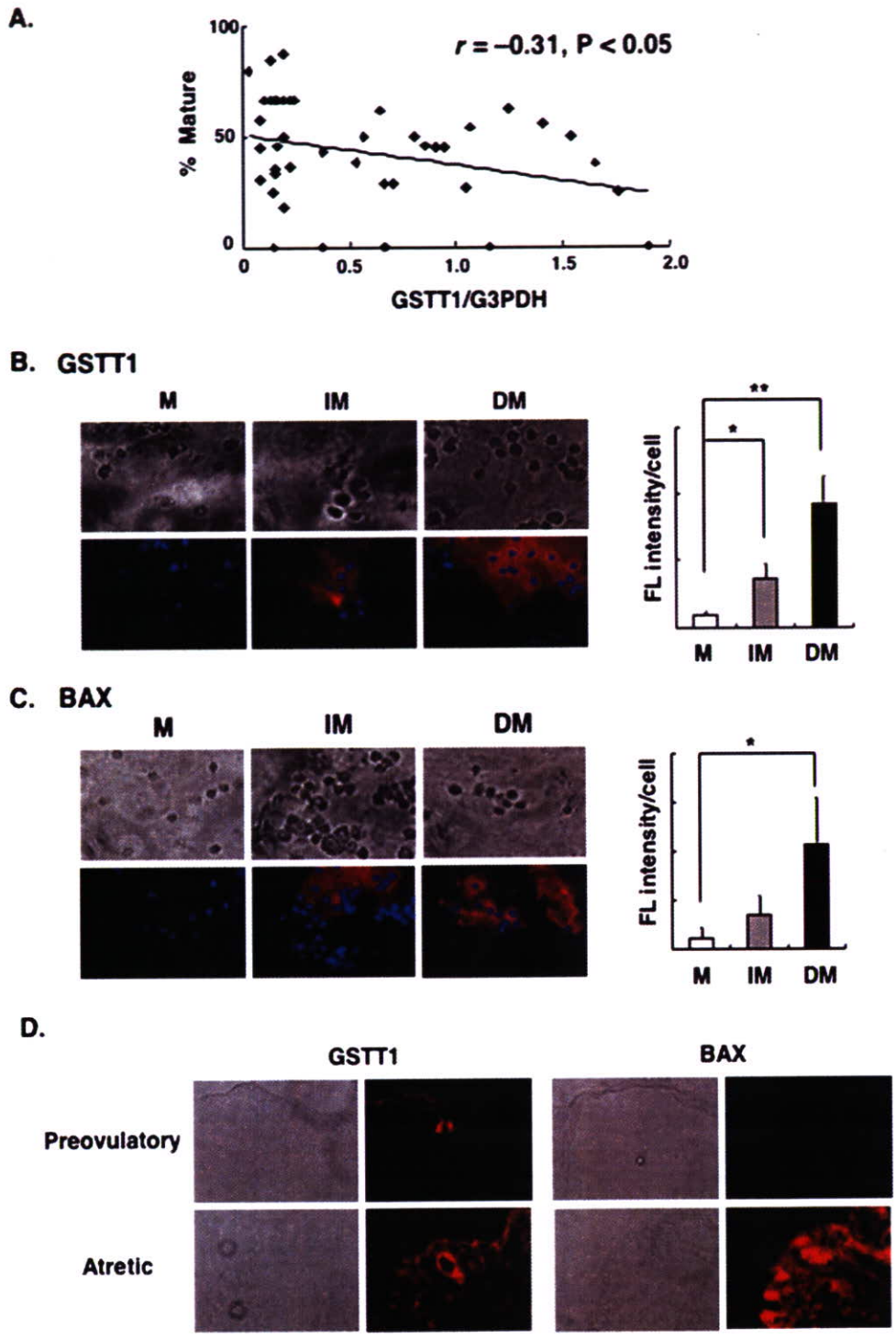
FIGURE 2



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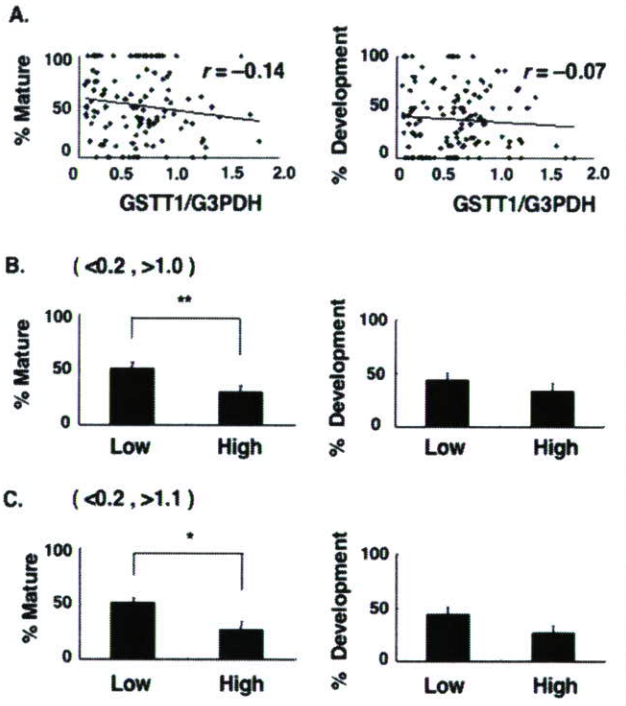
selected samples, the developmental capacity of oocytes from the patients with low GSTT1 was likely to be higher than that in the patients with high GSTT1 (Fig. 3B; low: 43.9%; high: 33.1%), although it was not significantly different (P=.3). The tendency was more apparent when high expression of GSTT1 was set at >1.1 GSTT1/G3PDH (Fig. 3C; COC maturity: low 51.3 %, N = 25; high 27.2%, N = 14, P<.05; developmental capacity: low 43.9 %; high 26.4%, P=.09). These

FIGURE 2 CONTINUED

Relationship between GSTT1 and COC maturity. (**A**) Correlation between GSTT1 mRNA expression in mural granulosa cells from male factor patients and frequency of mature COCs (N = 43). Statistical analysis was conducted using Pearson's correlation contingence ($r = -0.31$, $^*P<.05$). (**B, C**) Immunostaining of GSTT1 (**B**) and BAX (**C**) (red) in cumulus cells from male factor patients. *Left panels* show typical microphotographs of cumulus cells from mature (M), immature (IM), and dysmature (DM) COCs from a male factor patient (**B**: 35 years, **C**: 35 years). The cells were counterstained with Hoechst 33342 at 10 μM to visualize the nuclei (blue). *Right panels* show the mean fluorescence intensity of GSTT1 and BAX per cell with SEM from male factor patients (N = 5, 33–38 years). The data were analyzed by Student's or modified *t* test ($^*P<.05$, $^{**}P<.01$). (**D**) Immunostaining of GSTT1 and BAX in mouse preovulatory (8 hours post-hCG injection) and atretic follicles (72 hours post-PMSG injection).

FIGURE 3

Relationship between GSTT1 expression and oocyte quality in other factor patients. (**A**) Correlation between GSTT1 mRNA expression in mural granulosa cells from tubal and unknown factor patients and frequency of mature COCs or frequency of developmental capacity to the eight-cell stage (N = 126). Statistical analysis was conducted using Pearson's correlation contingence (not significant). (**B, C**) Frequency of mature COCs and developmental capacity of oocytes to eight-cell stage from tubal and unknown factor patients. GSTT1/G3PDH >1.0 (**B**, N = 17) or 1.1 (**C**, N = 14) was regarded as the high group and <0.2 (N = 25) as the low group. Those groups were compared statistically using Student's *t* test ($^*P<.05$, $^{**}P<.01$). Data are shown as the mean with SEM.



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results suggest that the definition of an optimal threshold of GSTT1 level may be useful for the diagnosis of age-related infertility.

FSH and Oxidative Stress as Inducers of GSTT1 in KGN Granulosa-Like Tumor Cells

In the process of aging, the serum concentration of gonadotropins such as FSH and LH is elevated (23). Thus, expression of GSTT1 in granulosa cells might be regulated by elevated gonadotropins. To examine this possibility, expression of GSTT1 in KGN cells stimulated with FSH, LH, and hCG was analyzed by immunostaining. FSH increased the expression of GSTT1, even at 0.5 ng/mL (Fig. 4A; $P<.05$), whereas LH and hCG did not (Fig. 4B, C). These results indicate that FSH could promote GSTT1 expression in granulosa cells. We next studied whether GSTT1 was up-regulated by oxidative stress in vitro, because oxidative stress is a major aging factor. When KGN cells were stimulated with H_2O_2 at 10 μM, GSTT1 was significantly up-regulated in a time-dependent manner (Fig. 4D; about 4.5-fold increase at 24 hours, $P<.001$), suggesting that oxidative stress was involved in the up-regulation of GSTT1. Taking these results together, our study supports the notion that GSTT1 is up-regulated by aging in granulosa cells and could be used as a biomarker for age-related infertility.

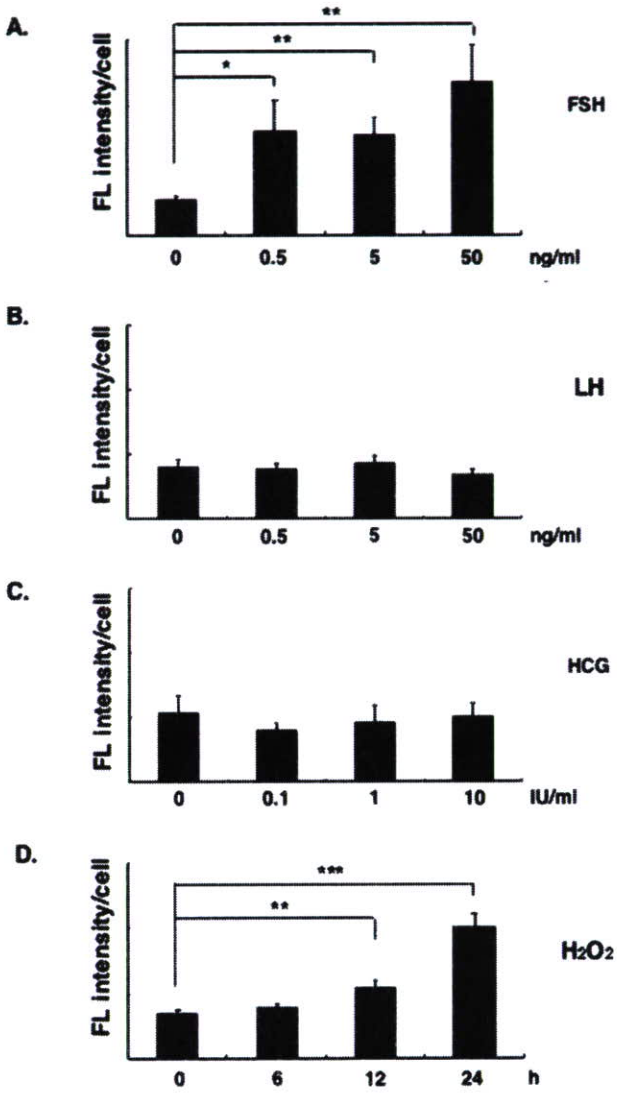
DISCUSSION

Reactive oxygen species and antioxidants are in balance in a young and healthy body; however, overabundance of ROS because of dysfunction of antioxidants during aging influences the reproductive life span of a woman (6). In fact, granulosa cells from older patients expressed less SOD1, SOD2, and catalase than those from young patients, and contained defective mitochondria (24). In addition, follicular fluid from older women exhibited decreased expression of GSTP and reduced activities of glutathione transferase and catalase (25). Although it is uncertain whether antioxidant systems are completely defective in aged reproductive cells, disorders of several molecules must be involved in accelerating reproductive aging.

GSTs are functionally versatile proteins. In addition to their function in catalyzing the conjugation of genotoxins to glutathione (GSH), peroxidase and isomerase activities have been reported. GSTs can also inhibit Jun N-terminal

FIGURE 4

Expression of GSTT1 in KGN cells treated with various stimuli. KGN cells were treated with recombinant human FSH (**A**), human LH (**B**), and hCG (**C**) at the indicated concentration for 12 hours, and with H_2O_2 at 10 μM for the indicated duration (**D**). The integrated fluorescence intensity of GSTT1 was measured and normalized with the number of cells. Data are shown as the mean fluorescence intensity per cell with SEM. Statistical analysis was conducted using one-way analysis of variance (**A**, $P<.05$; **D**, $P<.001$) followed with Student's *t* test (*$P<.05$, **$P<.01$, ***$P<.001$).



Ito. GSTT1 as a marker for age-related infertility. Fertil Steril 2007.

kinase, resulting in protection of cells against H_2O_2-induced cell death (26). In general, aging causes cellular dysfunction and apoptosis via loss of antioxidants; thus, GSTs are expected to be down-regulated by aging. In this study, GSTs, except for GSTT1, were down-regulated by aging in mural granulosa cells, although further analysis is required. It is of interest that GSTT1 could not bind to GSH-agarose or S-hexyl-GSH-agarose, unlike the other GSTs (27). GSTT1 has been reported to have conflicting properties, showing detoxification activity and production of a genotoxic metabolite (28). Therefore, the mechanism of expression of GSTT1 might be completely different from that of the other GSTs.

The information obtained from cumulus cells is important in predicting oocyte quality. Several indicators of oocyte quality have been reported using cumulus cells from infertile patients. For example, the abundance of pentraxin 3, a hyaluronan stabilizer, in cumulus cells was associated with the integrity of embryos leading to pregnancy (29). Similarly, cyclooxygenase, hyaluronic acid synthase 2, and gremlin were highly expressed in cumulus cells detached from oocytes scored as high grade (30). Reduced expression of progesterone receptors in cumulus cells was correlated with good embryo quality (31). Although the morphology of COCs was not associated with the nuclear maturity of oocytes (32, 33), our results showing a negative correlation between GSTT1 expression and COC maturity led to the idea that COC maturity might be consistent with the apoptotic status of cumulus cells. The apoptotic status of granulosa cells is altered with age (34), and correlates negatively with oocyte quality (8). In this context, the reported function of BAX in the acceleration of follicle atresia (35) and the expression pattern of BAX in cumulus cells in this study suggest that insufficiently mature COCs might be isolated from follicles towards atresia. Up-regulation of GSTT1 in granulosa cells might therefore be a response to apoptosis.

Diagnostic evaluation of oocyte quality is of clinical importance in achieving a successful pregnancy. Although morphologic evaluation of COCs can be useful as a first approach to the selection of oocytes of high quality, the second approach should use biomarkers. In this context, GSTT1 must be a predictor of both aging and oocyte quality. Although GSTT1 expression in the samples from tubal and unknown factor patients did not correlate with COC maturity, extraction of samples based on GSTT1 expression level gave an insight into the diagnosis of age-related infertility. A negative correlation between GSTT1 and COC maturity was observed in the selected granulosa cells from the tubal and unknown factor patients. Furthermore, the developmental capacity of oocytes from the selected patients with low GSTT1 was likely to be higher than that with high GSTT1. In this study, GSTT1 threshold was set at GSTT1/G3PDH >1 or 1.1 for high expression and GSTT1/G3PDH <0.2 for low expression to extract the aging factor from all other infertility factors. Because the mild selection of patients (e.g., <0.3 as low and >0.7 as high) did result in correlation with neither COC maturity nor developmental capacity (data not shown), a more severe selection seems to be more applicable for diagnosis of the aging factor. Although the additional biomarkers for oocyte quality might support the diagnosis, the GSTT1 level in granulosa cells could be a good indicator for age-related infertility.

The serum concentration of FSH and LH is known to increase gradually during aging (23). These changes in the ovarian micromilieu might affect the expression of downstream molecules. In our in vitro model, FSH appeared to induce GSTT1 expression. Interestingly, FSH is thought to act as an antiapoptotic molecule in ovaries and granulosa cells by increasing GSH (10, 36). A genetic and longitudinal study revealed that GSTT1 polymorphisms influence mortality in the elderly (37). Notably, hCG did not increase GSTT1 expression at any of the concentrations examined, although it has also been reported to possess an antiapoptotic activity (38). Up-regulation of GSTT1 might be regarded as a self-defense response downstream of FSH signaling.

Up-regulation of GSTT1 by oxidative stress in the in vitro model system may represent a self-defense response of granulosa cells against oxidative stress. Reactive oxygen species produced by oxidative stress act as important signaling molecules, whereas excess ROS diminish cellular functions. Exogenous application of oxidative stress could therefore evoke a self-defense mechanism, although the exact molecular mechanism of GSTT1 up-regulation was uncertain. However, several reports on increased apoptosis in granulosa cells during aging conflict with the antioxidant defense theory (34, 39). This discrepancy may be explained as follows. GSTT1 in reproductive cells is induced by increased oxidative stress and FSH during aging as a self-defense mechanism; however, oxidative stress may also trigger polymorphisms of GSTT1. As a result, overexpression of mutated GSTT1 (nonfunctional) could not rescue the cells. Further studies should be undertaken to evaluate this possibility.

In conclusion, the present study strongly suggests that GSTT1 in mural and cumulus granulosa cells is a possible indicator of aging of granulosa cells and oocytes. Both FSH and oxidative stress up-regulated GSTT1 in the granulosa cell line, supporting the notion that GSTT1 is a marker of aging. Notably, up-regulation of GSTT1 protein was related to the expression of BAX in cumulus cells classified as immature or dysmature, suggesting that the apoptotic status of cumulus cells is associated with the expression of GSTT1. Age-related decline of oocyte quality could be associated with oxidative stress-induced apoptosis defined by GSTT1 up-regulation.

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REFERENCES

1. Baird DT, Collins J, Egozcue J, Evers LH, Gianaroli L, Leridon H, et al. Fertility and ageing. Hum Reprod Update 2005;11:261–76.
2. Burwinkel TH, Buster JE, Scoggan JL, Carson SA. Basal follicle stimulating hormone (FSH) predicts response to controlled ovarian hyperstimulation (COH)-intrauterine insemination (IUI) therapy. J Assist Reprod Genet 1994;11:24–7.
3. te Velde ER, Scheffer GJ, Dorland M, Broekmans FJ, Fauser BC. Developmental and endocrine aspects of normal ovarian aging. Mol Cell Endocrinol 1998;145:67–73.
4. Pellestor F, Anahory T, Hamamah S. Effect of maternal age on the frequency of cytogenetic abnormalities in human oocytes. Cytogenet Genome Res 2005;111:206–12.
5. Linnane AW, Zhang C, Baumer A, Nagley P. Mitochondrial DNA mutation and the ageing process: bioenergy and pharmacological intervention. Mutat Res 1992;275:195–208.
6. Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. Reprod Biol Endocrinol 2005;3:28.
7. Chao HT, Lee SY, Lee HM, Liao TL, Wei YH, Kao SH. Repeated ovarian stimulations induce oxidative damage and mitochondrial DNA mutations in mouse ovaries. Ann N Y Acad Sci 2005;1042:148–56.
8. Seino T, Saito H, Kaneko T, Takahashi T, Kawachiya S, Kurachi H. Eight-hydroxy-2'-deoxyguanosine in granulosa cells is correlated with the quality of oocytes and embryos in an in vitro fertilization-embryo transfer program. Fertil Steril 2002;77:1184–90.
9. Das S, Chattopadhyay R, Ghosh S, Ghosh S, Goswami SK, Chakravarty BN, et al. Reactive oxygen species level in follicular fluid—embryo quality marker in IVF? Hum Reprod 2006;21:2403–7.
10. Tsai-Turton M, Luderer U. Opposing effects of glutathione depletion and follicle-stimulating hormone on reactive oxygen species and apoptosis in cultured preovulatory rat follicles. Endocrinology 2006;147:1224–36.
11. Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, et al. The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. Fertil Steril 1997;68:312–7.
12. Nakahara K, Saito H, Saito T, Ito M, Ohta N, Takahashi T, et al. Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. Fertil Steril 1998;69:931–5.
13. Mari M, Cederbaum AI. Induction of catalase, alpha, and microsomal glutathione S-transferase in CYP2E1 overexpressing HepG2 cells and protection against short-term oxidative stress. Hepatology 2001;33:652–61.
14. Deakin M, Elder J, Hendrickse C, Peckham D, Baldwin D, Pantin C, et al. Glutathione S-transferase GSTT1 genotypes and susceptibility to cancer: studies of interactions with GSTM1 in lung, oral, gastric and colorectal cancers. Carcinogenesis 1996;17:881–4.
15. Strange RC, Fryer AA. The glutathione S-transferases: influence of polymorphism on cancer susceptibility. IARC Sci Publ 1999;231–49.
16. Spurdle AB, Webb PM, Purdie DM, Chen X, Green A, Chenevix-Trench G. Polymorphisms at the glutathione S-transferase GSTM1, GSTT1 and GSTP1 loci: risk of ovarian cancer by histological subtype. Carcinogenesis 2001;22:67–72.
17. Seidegard J, Vorachek WR, Pero RW, Pearson WR. Hereditary differences in the expression of the human glutathione transferase active on trans-stilbene oxide are due tobecause of a gene deletion. Proc Natl Acad Sci USA 1988;85:7293–7.
18. Taningher M, Malacarne D, Izzotti A, Ugolini D, Parodi S. Drug metabolism polymorphisms as modulators of cancer susceptibility. Mutat Res 1999;436:227–61.
19. Wilson MH, Grant PJ, Hardie LJ, Wild CP. Glutathione S-transferase M1 null genotype is associated with a decreased risk of myocardial infarction. FASEB J 2000;14:791–6.
20. Sata F, Yamada H, Kondo T, Gong Y, Tozaki S, Kobashi G, et al. Glutathione S-transferase M1 and T1 polymorphisms and the risk of recurrent pregnancy loss. Mol Hum Reprod 2003;9:165–9.
21. Babu KA, Rao KL, Kanakavalli MK, Suryanarayana VV, Deenadayal M, Singh L. CYP1A1, GSTM1 and GSTT1 genetic polymorphism is associated with susceptibility to polycystic ovaries in South Indian women. Reprod Biomed Online 2004;9:194–200.
22. Nishi Y, Yanase T, Mu Y, Oba K, Ichino I, Saito M, et al. Establishment and characterization of a steroidogenic human granulosa-like tumor cell line, KGN, that expresses functional follicle-stimulating hormone receptor. Endocrinology 2001;142:437–45.
23. Klein NA, Battaglia DE, Fujimoto VY, Davis GS, Bremner WJ, Soules MR. Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J Clin Endocrinol Metab 1996;81:1038–45.
24. Tatone C, Carbone MC, Falone S, Aimola P, Giardinelli A, Caserta D, et al. Age-dependent changes in the expression of superoxide dismutases

and catalase are associated with ultrastructural modifications in human granulosa cells. Mol Hum Reprod 2006;12:655–60.
25. Carbone MC, Tatone C, Delle Monache S, Marci R, Caserta D, Colonna R, et al. Antioxidant enzymatic defences in human follicular fluid: characterization and age-dependent changes. Mol Hum Reprod 2003;9:639–43.
26. Sheehan D, Meade G, Foley VM, Dowd CA. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. Biochem J 2001;360:1–16.
27. Harris JM, Meyer DJ, Coles B, Ketherer B. A novel glutathione transferase (13-13) isolated from the matrix of rat liver mitochondria having structural similarity to class theta enzymes. Biochem J 1991;278:137–41.
28. Thier R, Pemble SE, Kramer H, Taylor JB, Guengerich FP, Ketterer B. Human glutathione S-transferase T1-1 enhances mutagenicity of 1,2-dibromoethane, dibromomethane and 1,2,3,4-diepoxybutane in *Salmonella typhimurium*. Carcinogenesis 1996;17:163–6.
29. 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(Suppl 1):1169–79.
30. 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.
31. Hasegawa J, Yanaihara A, Iwasaki S, Otsuka Y, Negishi M, Akahane T, et al. Reduction of progesterone receptor expression in human cumulus cells at the time of oocyte collection during IVF is associated with good embryo quality. Hum Reprod 2005;20:2194–200.
32. Hammitt DG, Syrop CH, Van Voorhis BJ, Walker DL, Miller TM, Barud KM. Maturational asynchrony between oocyte cumulus–coronal morphology and nuclear maturity in gonadotropin-releasing hormone agonist stimulations. Fertil Steril 1993;59:375–81.
33. Rattanachaiyanont M, Leader A, Leveille MC. Lack of correlation between oocyte–corona–cumulus complex morphology and nuclear maturity of oocytes collected in stimulated cycles for intracytoplasmic sperm injection. Fertil Steril 1999;71:937–40.
34. Sadraie SH, Saito H, Kaneko T, Saito T, Hiroi M. Effects of aging on ovarian fecundity in terms of the incidence of apoptotic granulosa cells. J Assist Reprod Genet 2000;17:168–73.
35. Perez GI, Robles R, Knudson CM, Flaws JA, Korsmeyer SJ, Tilly JL. Prolongation of ovarian lifespan into advanced chronological age by Bax-deficiency. Nat Genet 1999;21:200–3.
36. Tilly JL, Tilly KI. Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. Endocrinology 1995;136:242–52.
37. Christiansen L, Brasch-Andersen C, Bathum L, Kruse TA, Christensen K. A longitudinal study of the effect of GSTT1 and GSTM1 gene copy number on survival. Mech Ageing Dev 2006;127:597–9.
38. Matsubara H, Ikuta K, Ozaki Y, Suzuki Y, Suzuki N, Sato T, et al. Gonadotropins and cytokines affect luteal function through control of apoptosis in human luteinized granulosa cells. J Clin Endocrinol Metab 2000;85:1620–6.
39. Vaskivuo TE, Anttonen M, Herva R, Billig H, Dorland M, te Velde ER, et al. Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4. J Clin Endocrinol Metab 2001;86:3421–9.