The expression mechanism of glutathione S-transferase theta 1 (GSTT1) as an aging marker

（老化マーカー分子 glutathione S-transferase theta 1(GSTT1)の発現機構に関する研究）

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Contents

General introduction

Age-associated declines in female reproductive function become clinically evident during the late 30's. Reproductive aging has been reported to be accompanied by hormonal imbalance [1], decreased ovarian follicle pool [2], increased oocyte aneuploidy [3], and mitochondrial dysfunction in oocytes [4]. Although the exact mechanism of reproductive aging remains uncertain, an increasing amount of evidence suggests a probable involvement of reactive oxygen species (ROS) [5] generated inside or outside of the cells, especially in mitochondria, as one of the primary determinants of aging in general [6]. ROS are known to act as important signalling molecules. However, they inversely damage cellular components and cytoplasmic organelles. Young and healthy cells possess an intact antioxidant defense system and immediately remove excess ROS to protect themselves, whereas aging causes attenuation or loss of their protective ability, resulting in cell death.

Several candidate biomarkers for oocyte quality have been reported. Follicular fluid is thought to contain markers for oocyte quality [7]. ROS present in the follicular fluid may act as indicators of embryo quality; ROS levels in follicular fluid containing oocytes graded with a low score were significantly high as compared with those in follicular fluid containing oocytes with a high score [8]. Also, high concentrations of lactoferrin and anti-Müllerian hormone in follicular fluid were

reported to reflect high quality of oocytes[9]. In granulosa cells, 8-hydroxy-2'-deoxyguanosine (8-OHdG) produced by oxidative stress was reported to correlate negatively with the quality of oocytes and embryos[10]. In addition, higher expression of HAS2, PTGS2 and GREM1 in cumulus cells was reported to correlate positively with good embryo quality[5]

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[11]. Substrates for GSTs include many environmental pollutants, pesticides, antibiotics, antineoplastics and carcinogenic products of phase I metabolism. Biochemical functions of GST enzymes include catalysing the addition of glutathione (GSH) to electrophilic xenobiotics, facilitating the transfer of reducing equivalents to toxic products generated during oxidative stress, and chemical sequestration [12,13]. Cytosolic GST isoenzymes are composed of two subunits that exist as homo- or hetero-dimers and are encoded by five distantly related gene families designated as class Alpha, Mu, Pi, Sigma and Theta [14-16]. Microsomal forms of GSTs exist as trimers and are encoded by a separate gene. Individual isoenzymes display distinctive catalytic properties and marked substrate

specificities. Biological mechanisms responsible for controlling the expression and regulation of GST isoenzymes are complex and not well characterized. Individual isoenzymes are induced by an extensive range of xenobiotics, regulated by hormones and expressed in a manner specific to gender, species, tissue, age and tumour status [17-21].

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[22-24]. 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[25]. The mutated products modulate chemical binding to DNA, and are associated with myocardial infarction as well as the tobacco-related cancers in smokers[26,27]. Also, polymorphisms of GSTM1 and GSTT1 may increase the risk of recurrent pregnancy loss [28], and susceptibility to polycystic ovaries[29]. Because of the limited availability of molecular information and biomarkers for age-related infertility, effective diagnosis and therapy have not yet been established.

We have recently reported that GSTT1 expressed in granulosa cells is a biomarker for the maturity of cumulus-oocyte complexes in age-related infertility. It

was shown to be up-regulated by aging in granulosa cells and correlated negatively with the maturity of cumulus-oocyte complexes[30]. Despite these efforts to identify biomarkers of oocyte quality, only few have been found for age-related infertility, as age-related infertility has not become an issue until recently. The measurement of the GSTT1 level in the granulosa cells binding to patient's oocytes could be used as the selection of oocyte of high quality, meaning that the GSTT1 level in granulosa cells could be a good indicator for age-related infertility. It is necessary to confirm the relationship between aging and GSTT1 up-regulation. However, the regulation mechanism of GSTT1 in granulosa cells remains unclear. GSTs are classified as phase II detoxification enzymes and known to be regulated by nuclear factor erythroid 2 p45-related factor 2 (Nrf2), a transcription factor in general[31,32]. Nrf2 is a DNA-binding protein for antioxidant response element (ARE) of the promoter regions and activated by oxidative stress and electrophiles, regulating the expression of numerous detoxification and antioxidant gene, including GSTs[33,34]. However, it remains unclear that GSTT1 is regulated by Nrf2-ARE pathway in the same manner as other GSTs.

In the present study, I attempted to examine the difference of the expression and regulation mechanism between GSTT1 and other GSTs.

Chapter 1

GSTT1 is a marker for aged granulosa cells and may have different regulation system from other GSTs

Abstract

Glutathione S-transferases (GSTs) are known to detoxify the metabolites of genotoxic molecules to more water-soluble and readily excretable forms. We recently demonstrated that GSTT1 is expressed in granulosa cells as a biomarker for oocyte quality in age-related infertility. However the regulation mechanism of GSTT1 in granulosa and other somatic cells remains unclear. In the present study, I attempted to examine the difference of the expression mechanism between GSTT1 and other GSTs. First, the expression patterns of GSTT1 and other GSTs in aged granulosa cells from patients and mice were examined by RT-PCR and immunostaining. Although other GSTs were down-regulated in aged samples, GSTT1 was up-regulated. GSTs are known to be regulated nuclear factor erythroid 2 p45-related factor 2 (Nrf2), a transcription factor for antioxidative molecules. I next analyzed the expression patterns of GSTs in Nrf2 gene disrupted mice (Nrf2KO). The expressions of GSTs except for GSTT1 were decreased in MEFs from Nrf2KO mice. On the other hand, GSTT1 was not changed in those of mice. These results suggest that the expression of GSTT1 is not regulated by Nrf2 differently from other GSTs.

Introduction

Maternal age is a risk factor for infertility. The decline in fecundity becomes clinically evident when women reach their mid-30s[35]. Although the exact mechanism by which aging causes female reproductive disorders is unclear, age-related changes in the ovary including hormonal imbalance[1], decrease of the ovarian follicle pool[36], increase of oocyte anueuploidy, and mitochondrial dysfunction in oocytes[3] might 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. In addition, oocytes and somatic cells stored in ovaries are thought to be exposed to reactive oxygen species (ROS) during both ovulation and aging[37]. In general, antioxidants which scavenge ROS and protect cells from oxidative stress-induced cell death are decreasing with ageing [38,39].

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[11]. In this regard, most of all GSTs may be down-regulated by aging. We recently

demonstrated that only GSTT1 was up-regulated in granulosa cells from aged patients and aged mice although other kinds of GSTs were down-regulated in these cells. The measurement of the GSTT1 level in the granulosa cells binding to patient's oocytes could be used as the selection of oocyte of high quality, meaning that the GSTT1 level in granulosa cells could be a good indicator for age-related infertility[30]. However, the regulation mechanism of GSTT1 remains unclear.

GSTs are classified as phase II detoxification enzymes and known to be regulated by nuclear factor erythroid 2 p45-rerated factor 2 (Nrf2), a transcription factor in general[31,32]. Nrf2 is a DNA-binding protein for antioxidant response element (ARE) of the promoter regions and activated by oxidative stress and electrophiles, regulating the expression of numerous detoxification and antioxidant genes, including GSTs[33,34]. Targeted disruption of Nrf2 in mice (Nrf2KO) have been reported to show decreased expression of most GSTs and the catalytic subunit of glutamate cysteine ligase (GCLC) as compared to wild type mice[31,32,40,41]. Shih et al. have reported that age-related declines of antioxidant defense are closely related the expression of Nrf2 in rats[42], suggesting that Nrf2 is thought to be down-regulated with aging as well as antioxidants including GSTs.

Therefore, in the present study, I attempted to examine the difference of

the expression mechanism between GSTT1 and other GSTs using granulosa cells from aged patients and mice, and Nrf2KO mice.

Materials and Methods

Reagents

Hoechst 33342, human FSH, CDNB (1-Chloro-2,4-dinitro-benzene), and DCNB (3,4-Dichloronitro-benzene) were purchased from Sigma Chemical Co (St. Louis, MO). DCM (Dichloromethane) and Trichloroacetic Acid Solution were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). SP600125 was purchased from Biomol International, L.P. (Pymouth meeting, PA). Rabbit polyclonal antibodies against GSTT1 and Nrf2, and GSTP1 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA) and Stressgen Bioreagents Co. (Ann Arbor, MI) respectively. Goat polyclonal antibody against GSTA was purchased from Abcam Co. (Tokyo, Japan). Alexa Fluor 488 conjugated goat anti-rabbit IgG and donkey anti-goat IgG antibodies were purchased from Molecular Probes, Inc. (Eugene, OR, USA).

Patients and samples

Mural granulosa cells were obtained from women undergoing in vitro fertilization (IVF)/intracytoplasmic sperm injection cycles between August 2003 to December 2007 at the National Center for Child Health and Development, Japan. Thirty four women participated in this study. The local ethics committee approved the

experiments on these samples and all individual women provided prior informed consent.

Mural and cumulus granulosa cells were isolated from follicular aspirates, and washed three to four times in PBS containing 1 mg/ml BSA (PBS+BSA) immediately after aspiration. Cumulus-oocyte complexes (COCs) were also isolated and washed twice in IVF medium (HTF medium purchased from Irvine Scientific Co., Santa Ana, CA, USA). Cumulus cells were then detached from COCs physically with 27G fine needles in IVF medium, and washed three times in PBS + BSA. A portion of the mural and cumulus granulosa cells was transferred into 1.5 mL microtubes, centrifuged for 5 min 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 min at room temperature, washed three times in PBS + BSA, put on glass slides, and air-dried, and then stored at 4°C until use.

Enzyme assays

Glutathione S-transferase activities toward CDNB and DCNB were measured by the spectrophotometric methods published by Habig *et al* with some modifications[43]. One milliliter of the reaction buffer containing 1 mM CDNB or

DCNB, 1 mM GSH, 0.1M potasium phosphate (pH 6.5 or pH 7.5) was mixed with cytosolic protein and incubated for 30 min at 25°C. Then the rates of S-(2,4-dinitrophenyl)glutathione and S-(2-chloro-4-nitrophenyl)glutathione formation were determined by measuring the absorption at 340 nm and 345 nm, respectively (340 nm = 9.6 mM cm^{-1}, 345 nm = 8.5 mM cm^{-1}). Glutathione S-transferase theta 1 (GSTT1) activity toward DCM was determined with the colorimetric method reported by Nash (1953) with some modifications. One milliliter of reaction buffer (10 mM GSH, 20mM TRIS/HCl, pH7.4) was mixed with cytosolic protein and 4 μl DCM and incubated for 30 min at 37°C. One milliliter of this reaction was mixed with 20% of trichloroacetic acid and centrifuged for 2 min at 16,000x*g*. One milliliter of the supernatant was mixed with one milliliter of Nash-reagent and incubated for 1 h at 37°C. Then the rate of HCHO formation was determined by measuring the absorption at 412 nm. All measurements were corrected for non-enzymatic conjugation rates. Enzyme activity is expressed as μmol/mg of protein/min. The values were the means from at least three independent experiments.

Animals and preparation of mouse oocytes and ovaries

Female C57BL/6J mice were purchased from Japan SLC, Inc. (Tokyo,

Japan). Nrf2-disrupted C57BL/6J mice were produced by Yamamoto et al and obtained from Riken Bioresouce[44]. They were superovulated by intraperitoneal injections of 5~6 IU PMSG followed by 5~6 IU hCG 48 h later. The ovulated COCs were then recovered from oviducts at 13–14 h post hCG injection, and fixed with 4% paraformaldehyde in PBS + BSA for 20 min, washed three times in PBS + BSA and stored at 4°C until use. A part of ovaries were fixed in 4% paraformaldehyde in PBS for 20 min. They were then sectioned serially by cryostat (8 μm interval, Reica), and subjected to immunofluorescence studies. The sections were kept at 4°C until use.

All animal care and experimentation were conducted in accordance with the guidelines of The University of Tokyo. All the experiments were conducted with an accreditation of the Animal Care and Use Committee of the Graduate School of Agriculture and Life Sciences, the University of Tokyo.

Preparation of mouse embryonic fibroblasts (MEFs)

MEFs were isolated from mouse embryos at day 14.5. The embryos are dissociated and then trypsinized to produce single-cell suspensions. A portion of the cells was transferred into 1.5 mL microtubes, centrifuged for 5 min at 15,000 rpm at 4℃ to remove excess buffer, and stored at –80°C until use. The remaining samples were

seeded and cultured into 8-well culture slides (BD Japan Co. Ltd., Tokyo, Japan) for immunofluorescence study.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from mouse COCs and MEFs 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, USA), according to the manufacturer's instructions as described previously [45]. The expressions of mRNA were then monitored by RT-PCR analysis. The primer sets used in this experiment were described in Table 1. The conditions for PCR amplification were as follows: initial denaturation at 94°C for 2 min, followed by gene specific cycles of denaturation at 94°C for 45 sec, annealing at 57°C for 45 sec and elongation at 72°C for 1 min with a final extension at 72°C for 15 min.

The PCR products were separated on 2% agarose gel and stained with ethidium bromide. The digital photographs were taken on a transilluminator, and saved as JPEG files. The averaged band intensity was measured using Adobe Photoshop Element 3.0 software and the background noise was subtracted. Semi-quantitative data

on the expression level of each gene were obtained by comparison with expression levels of β-tubulin and G3PDH.

Immunofluorescence examination

Mural and cumulus granulosa cells and MEFs prepared as described above were permeabilized with 0.1% Triton X-100 in PBS for 30 min at room temperature. The COCs obtained from mice were also permeabilized with 1% Triton X-100 in PBS for 30 min at room temperature. These samples were blocked with 100% Block Ace (Snow Brand Milk Products Co., Tokyo, Japan) overnight at 4°C. Then they were treated with the first antibody at 20 μg/ml (for GSTT1 and Nrf2) or 5 μg/ml (for GSTA and GSTP) overnight at 4°C, washed three times with PBS, and visualized with goat anti-rabbit IgG conjugated with Alexa Fluor 488 and donkey anti-goat IgG conjugated with Alexa Fluor 488 diluted at 1:1,000 in PBS + 0.1% Triton X-100 for 1 h at room temperature. To count the number of cells, Hoechst 33342 was also loaded at 10 μM in PBS+0.1% Triton X-100 during treatment with the secondary antibody. They were then washed three times with PBS, immersed with VectaShield (Vector Laboratories Inc., Burlingame, CA) and covered with coverslips. Microphotographs were taken under an epifluorescence microscope equipped with a computational CCD

camera (Olympus, Tokyo, Japan). For image analysis, photographs were taken from 5 different areas in each sample with MetaMorph software (Molecular Devices Corp. Tokyo, Japan), and the averaged fluorescence intensity in each image was measured. The averaged 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 mRNA and protein expressions and enzyme activities are shown as the means with standard error of means (SEM) from at least three independent experiments. These data were analyzed with Student's *t*-test or modified *t*-test (Welch's correction). Differences were considered statistically significant when $P<0.05$. These analyses were performed using Microsoft Excel software.

Results

Up-regulation of GSTT1 differentially from other GSTs in granulosa cells with aging

We recently demonstrated that GSTT1 was up-regulated in granulosa cells from aged patients[30] although GSTP, a one of major class of GSTs was down-regulated in these cells(Fig. 1-1). Therefore the enzymatic activities for GSTs were measured by using the GST-specific substrates, CDNB and DCNB, except for GSTT1 and the GSTT1-specific substrate in granulosa cells from young and aged patients. Expectedly most GSTs activities were more decreased in aged than young. On the other hand, GSTT1 activity was not so, though the significant difference was not observed (Fig. 1-2). Next I examined whether similar result was obtained from mouse granulosa cells and analyzed GSTT1 and other GSTs from young (8~9 weeks) and aged (50~52 weeks) mouse granulosa cells. As with the result of human granulosa cells, GSTT1 was found to be up-regulated in aged (Fig. 1-3;$P<0.05$). However, GSTA1 and GSTM1 were down-regulated, and GSTP1 was not different (Fig. 1-3; GSTA1, GSTM1 $P<0.05$). Similar results were obtained from immunofluorescence studies. GSTT1 protein expression was also up-regulated by aged granulosa cells (Fig. 1-4). These results demonstrate that unlike other GSTs, GSTT1 tend to be up-regulated with aging and could be used as a biomarker for aged granulosa cells from not only patients but

also mice.

Different expression pattern of GSTT1 from other GSTs in mouse embryonic fibroblasts (MEFs) from Nrf2- disrupted mice.

Previous study showed that GSTT1 expression pattern was different from others (data not shown). In order to clarify that in detail, I analyzed the relationship between GSTT1 and Nrf2, a transcription factor for GSTs by using Nrf2 gene-disrupted mice. So far, Nrf2KO mice are well known to be reduced expressions of GSTs in various tissues[31,32,40,46]. I prepared mouse embryonic fibroblasts (MEFs) from WT and Nrf2KO mice, and compared the expression of GSTT1, GSTP1 and GSTA1 by semi-quantitative RT-PCR analysis. As with previous reports, mRNA expressions of GSTA1 were significantly down-regulated in MEFs from Nrf2KO mice in compared to those of WT; though GSTT1 did not make significant difference (Fig. 1-5). In addition, immunofluorescence studies also showed significant difference in GSTP (Fig. 1-6). Ultimately, these results indicated that the Nrf2 mediated-expression of GSTT1 is slim to none unlike other GSTs.

Discussion

Reactive oxygen species (ROS) 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[37]. In fact, granulosa cells from older patients expressed less SOD1, SOD2, and catalase than those from young patients, and contained defective mitochondria[39]. In addition, follicular fluid from older women exhibited decreased expression of GSTP and reduced activities of glutathione transferases and catalases[47]. 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 play a critical role in antioxidants system and protect from ROS by inactivating them and repairing cell damage[48]. It has been suggested that age-related decline of competence of fertility are influenced with ROS[35,37]. In general, aging causes cellular dysfunction and apoptosis via loss of antioxidants; thus, GSTs are expected to be down-regulated by aging[49,50]. However, in this study granulosa cells from aged patients showed reduced activities of GSTs expect for GSTT1. Aged mice also showed less expression of GSTs than those from young. Previously we reported that a negative correlation between GSTT1 expression and COC maturity, and that is

consistent with the apoptotic status of cumulus cells. Furthermore, the expression pattern of Bax, an apoptosis marker, was correlated with that of GSTT1 in cumulus cells[30]. In this context, up-regulation of GSTT1 in granulose cells might be associated in some way with age-related apoptosis.

GSTs are classified as phase II detoxification enzyme and believed to be regulated by Nrf2, a transcription factor. Nrf2 is known to be activated by oxidative-stress, electrophiles, many chemicals and cancer chemopreventive agents, and regulates the expression of various detoxifying and antioxidant genes including GSTs[33,34]. My results showed MEFs from Nrf2-disrupted mice expressed less GSTs than those of WT mice with the exception of GSTT1. On the other hand, the expression level of GSTT1 was not changed, indicating that the stress response for GSTT1 might be different from other GSTs.

Table 1-1

Primers sets for semi-quantitative RT-PCR

genes	Foreword primer (5'-3')	Reversed primer (5'-3')
Human GSTT1	GGATCTGATTAAAGGTCAGCACTTA	ACCAGTAGTCAGGGACCTTATATTT
Human GSTP1	ATCTCCCTCATCTACACCAACTAT	AGTCCAGCAGGTTGTAGTCAG
Human Nrf2	GCCCACATTCCCAAATCAGAT	CGTAGCCGAAGAAACCTCATTG
Human GSTA1	GATTTGTTTTCATTAGGATCTGA	CATGGAGAAGATTGGAAATCTGAAT
Human GSTM1	AAGCTATGAGGAAAAGAAGTACAC	ATAGACGAGAAAATCTACAAAAGTG
Human GATA-4	CTCCTTCAGGCAGTGAGAGCC	GGTCCGTGCAGGAATTTGAGG
Human GATA-6	TTCTAACTCAGATGATTGCAG	GCTGCACAAAAGCAGACACGA
Human G3PDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA
Mouse GSTT1	CTTCGCCAAGAAGAACAATATC	AGTCTTTGTCCTGGAGGAACT
Mouse GSTP1	CCTTGGCCGCTCTTTGG	GGCCTTCACGTAGTCATTCTTACC
Mouse Nrf2	GCCCACATTCCCAAATCAGAT	CGTAGCCGAAGAAACCTCATTG
Mouse GSTA1	ACAGGTCACTACCTGTGCAATGCC	CCTGACAGTACCACAGGTCCTAG
Mouse GSTM1	AGCACCACCTGGATGGAG	AGTCAGGGTTGTAACAGAGCAT
Mouse Bax	AAGCTGAGCGAGTGTCTCCGGTG	GCCACAAAGATGGTCACTGTCTGCC
Mouse Bcl2	CTCGTCGCTACCGTCGTGACTTCG	CAGATGCCGGTTCAGGTACTCAGTC
Mouse GATA-1	ATGGATTTTCCTGGTCTAGGGGC	TCAAGAACTGAGTGGGGCGATCACG
Mouse GATA-4	GGAAGACACCCCAATCTCG	CATGGCCCCACAATTGAC
Mouse GATA-6	ACCTTATGGCGTAGAAATGCTGAGGGTG	CTGAATACTTGAGGTCACTGTTCTCGGG
Mouse B-tublin	CAGGCCGGACAGTGTGGCAAC	GGCTTCATTATAGTACACAGAGATTCG

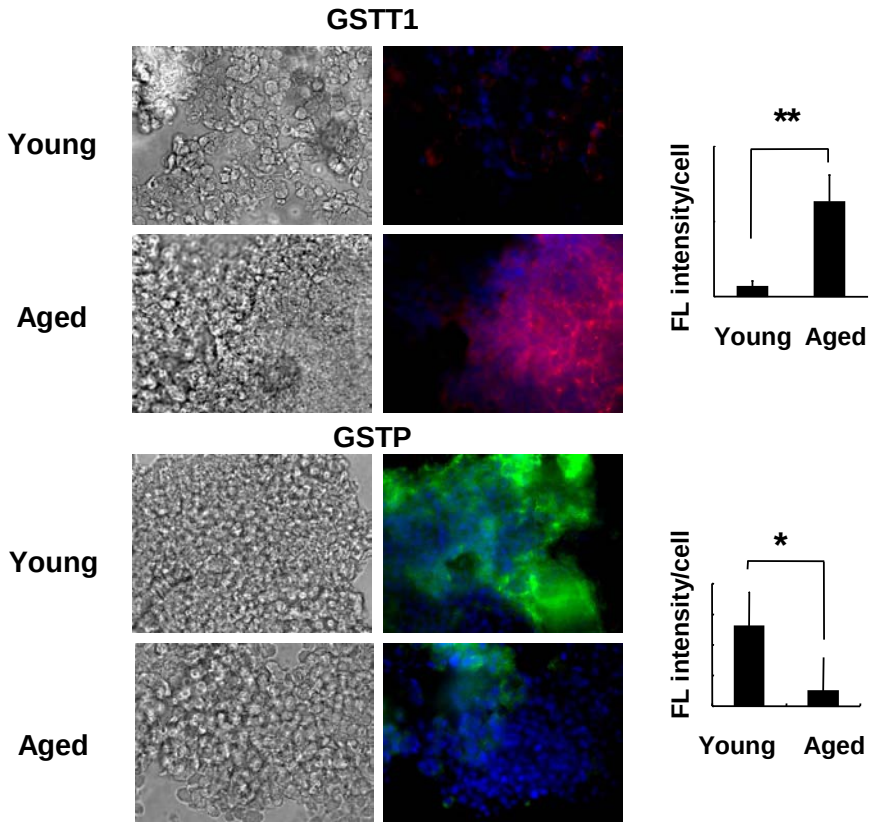


Fig. 1-1

The expression analysis and the enzyme assays for GSTT1 and other GSTs in granulosa cells from patients

Immunostaining of GSTT1 protein (red) and GSTP protein (green) in granulosa cells from young (25-34 years old) and aged (38-43 years old) patients (GSTT1; young N=9 vs aged N=12, GSTP; young, N=5 vs aged, N=7). Left panels show typical microphotographs from young and aged granulosa cells. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue). Right panels show the mean fluorescence intensity of GSTT1 and GSTP per cell with SEM from young and aged granulosa cells. Statistical analysis was conducted using Student's *t*-test (**P < 0.01, *P < 0.05).

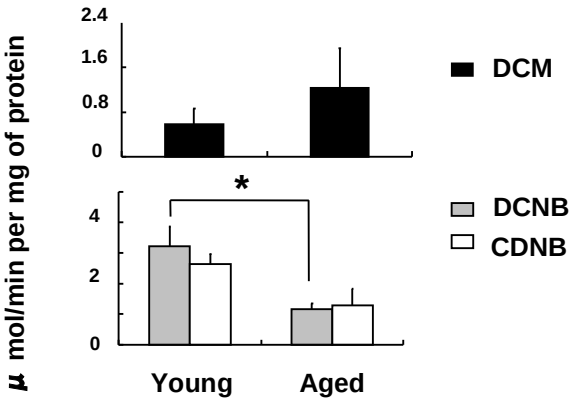


Fig. 1-2

The expression analysis and the enzyme assays for GSTT1 and other GSTs in granulosa cells from patients

The enzyme activity of GSTs toward DCM for GSTT1-specific substrate and CDNB, DCNB for other GSTs substrate in aged granulosa cells (Fig.1B, young: 25-27 years, N=5, aged: 35-41years, N=7 $P<0.05$). Statistical analysis was conducted using Student's *t*-test (*$P < 0.05$).

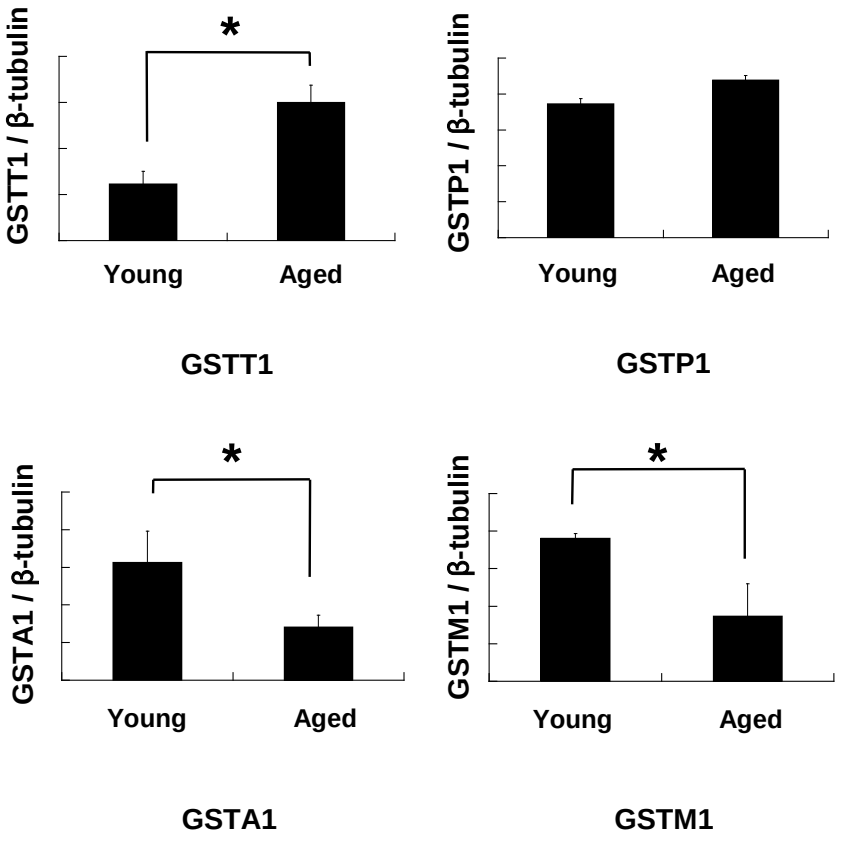


Fig. 1-3

The expression analysis for GSTT1 and other GSTs in granulosa cells in aged mice

Semi-quantitative RT-PCR analysis of GSTs in young 8 weeks of and aged 50 weeks of mice. Total RNA were isolated from cumulus granulosa cells, and RT-PCR analysis was performed using mouse specific primers for GSTs. These graphs represent the averaged band intensity of GSTs with SEM, normalized with β-tublin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

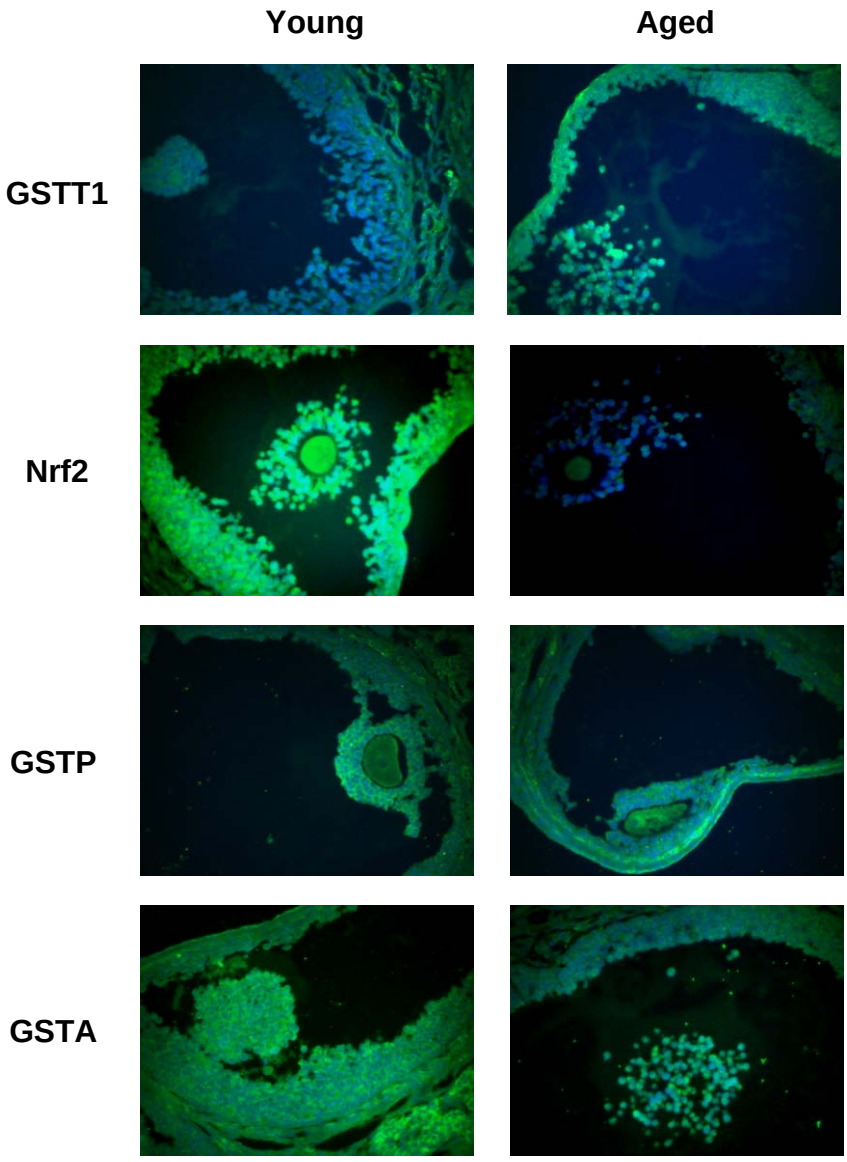


Fig. 1-4

The expression analysis for GSTT1 and other GSTs in granulosa cells in aged mice

Immunostaining of GST proteins (green) in ovarian follicles from young 8 weeks of and aged 50 weeks of mice. Theses panels show typical microphotographs in these samples. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue).

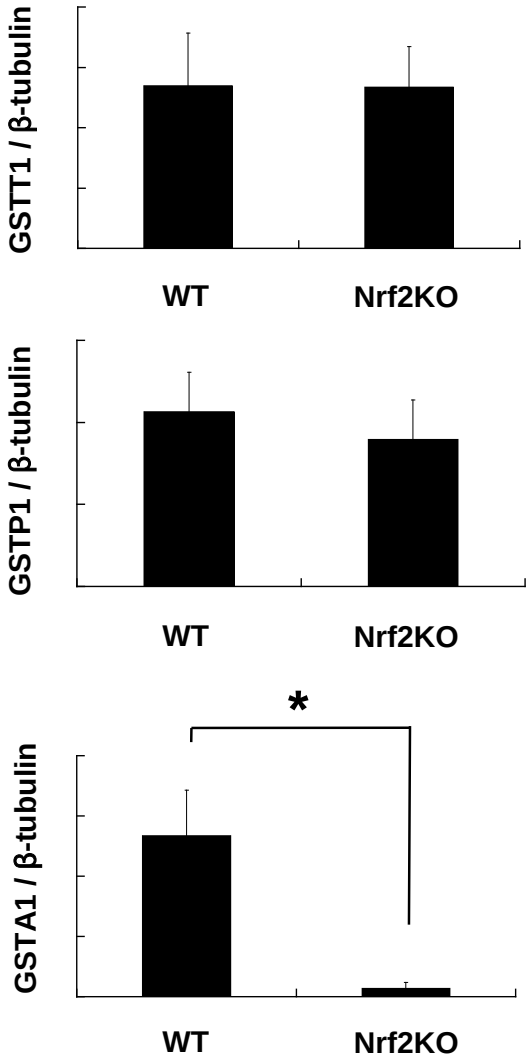


Fig. 1-5

The expression pattern of GSTT1 and the other GSTs in mouse embryonic fibroblasts (MEFs) from Nrf2-disrupted mice

Semi-quantitative RT-PCR analysis of GSTs in MEFs from WT and Nrf2KO mice. The total RNA were isolated from MEFs in these mice, and RT-PCR analysis was performed using mouse specific primers for GSTs. Theses graphs represent the averaged band intensity of GSTs with SEM, normalized with β-tublin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

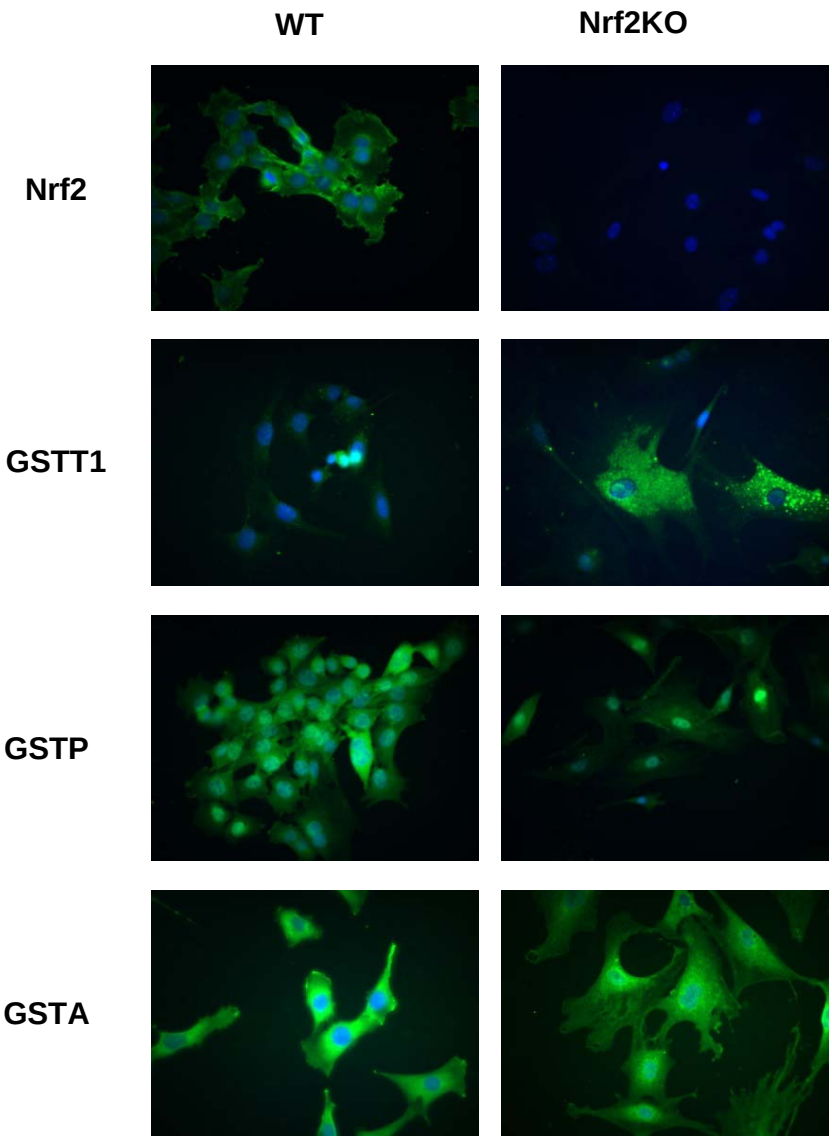


Fig. 1-6

The expression pattern of GSTT1 and the other GSTs in mouse embryonic fibroblasts (MEFs) from Nrf2-disrupted mice

Immunostaining of GSTT1, Nrf2, GSTP and GSTA protein (green) in MEFs from WT and Nrf2KO. Theses panels show typical microphotographs from them. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue).

Chapter 2

GSTT1 expression is induced in KGN cells by FSH and H_2O_2 stresses via differential stress kinase from other GSTs

Abstract

Taking previous results into consideration, I focused on the transcriptional regulation of GSTT1. Since previous experiment showed that GSTT1 was induced by FSH stimuli in human granulosa cell line (KGN cells), I examined whether similar results were obtained in GSTs or not. Interestingly only GSTT1 was induced by FSH. Next, to examine whether GSTT1 was induced by Nrf2 or not KGN cells was treated with all-trans retinoic acid (ATRA), Nrf2 inhibitor for GST expression analysis. ATRA inhibited GSTA, GSTP and GSTM expressions, while GSTT1 was not. In addition, I examined the effect of other stress kinase inhibitor SB203580 and SP600125, JNK and p38 MAPK inhibitors, respectively. As a result, GSTT1 expression was inhibited by p38 MAPK inhibitor, unlike other GSTs, indicating that GSTT1 might be induced by a different stress response from other GSTs.

Introduction

I previously demonstrated that GSTT1 was up-regulated with aging in granulosa cells from human and mice, and MEFs from Nrf2 gene-disrupted mice were not changed in GSTT1 expression, unlike other GSTs. Therefore I predicted that GSTT1 might have different stress response from others.

Phase II detoxification enzyme genes is coordinately up-regulated in response to oxidative stress, and this involves the activation of an electrophile response element (EpRE) in their promoter regions[51]. The activation of EpRE is triggered primarily by the binding of a transcription factor, Nrf2. Under resting condition, Nrf2 is retained in the cytosol by associating with the Kelch-like ECH-associated protein 1 (Keap1). Upon exposure to electrophilic compounds, especially those producing oxidative stress, Nrf2 is released from Keap1 and translocated into the nucleus, where it forms heterodimers with other transcription factors such as Jun family proteins and binds to EpRE to promote the transcription of phase II detoxification enzyme genes [52-54](Fig. 2-1).

Despite the common downstream EpRE–Nrf2 pathway, a variety of upstream signaling pathways have been reportedly implicated in the induction of phase II detoxification genes. These include extracellular signal-regulated kinase (ERK), p38

mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K). Even for the induction of a single gene, various upstream signaling pathways are involved[55]. It is assumed that the upstream signaling pathways involved in the induction of Nrf2–EpRE-regulating genes may be affected by gene, inducer and cell type. Among these kinases, ERK is mainly activated by mitogens and growth factors, while p38 MAPK and JNK are activated by many environmental stress stimuli, including oxidative stress defense response[56,57]. GSTP1 is involved in cellular proliferation pathways and has been proposed that monomeric GSTP1 functions as a JNK inhibitor[48,58]. Also, various stimuli such as UV light, osmotic shock, inflammatory cytokines, and growth factors can induce p38 MAPK signaling, and strong linkage between p38 MAPK signaling and aging has been shown in various tissues and organs[59-63]. In the present study, I examined whether or not GSTT1 expression interacts with JNK or p38 MAPK pathway. Additionally, it is reported that all-trans retinoic acid (ATRA), and other retinoic acid receptor alpha (RARα) agonists, markedly reduces the ability of Nrf2 to mediate induction of ARE-driven genes by cancer chemopreventive agents including the metabolites of butylated hydroxyanisole, tert-butylhydroquinone (tBHQ), and in MCF7 cells, ATRA did not block the nuclear accumulation of Nrf2 but reduced the binding of

Nrf2 to the ARE[64]. Therefore, to clarify the relationship between GSTT1 and Nrf2, I examined the effect of ATRA and other stress kinase inhibitor SB203580 and SP600125, JNK and p38 MAPK inhibitors, respectively in KGN cells.

Materials and Methods

Reagents

FSH and all-trans retinoic acid (ATRA) were purchased from Sigma Chemical Co (St. Louis, MO). SB203580 and rabbit polyclonal antibodies against Bax were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA). SP600125 was purchased from Biomol International, L.P. (Pymouth meeting, PA).

Cell culture and treatment

Human granulosa-like tumor cell line KGN was used [65]. Cells were maintained in a Dulbecco's modified Eagle's medium (DMEM) /Ham's F12 medium (Sigma) 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. Cells were seeded and cultured into 10 cm dish or 8-well culture slides (BD Japan Co. Ltd., Tokyo, Japan) for immunofluorescence study. Cells were stimulated with FSH, ATRA (1 μM) or oxidative stress (H_2O_2, 300 μM) with or without SB203580 and SP600125 (each 10 μM) for 24 h. The medium was replaced 2 h before treatment of cells.

Immunofluorescence examination

The immunofluorescence staining method of KGN cells was described in Chapter 1. They were treated with the first antibody at 20 μg/ml (for Bax) overnight at 4°C, washed three times with PBS, and visualized with goat anti-rabbit IgG conjugated with Alexa Fluor 488 diluted at 1:1,000 in PBS + 0.1% Triton X-100 for 1 h at room temperature as described in Chapter 1.

Semi-quantitative RT-PCR analysis

Reverse transcription using total RNA isolated from human mural granulosa cells was performed as described in Chapter 1. The expressions of mRNA were then monitored by RT-PCR analysis. The primer sets used in this experiment were described in Chapter 1 (Table 1-1).

Statistical analysis

Normalized data on the mRNA and protein expressions are shown as the means with standard error of the means (SEM) from at least three independent experiments. These data were analyzed with Student's *t*-test or modified *t*-test (Welch's correction). Differences were considered statistically significant when $P<0.05$. These

analyses were performed using Microsoft Excel software.

Results

Only GSTT1 was induced by FSH unlike other GSTs

In the process of aging, the serum concentration of gonadotropins such as FSH, LH and oxidative stress are elevated[66]. Our previous experiment showed that FSH increased the expression of GSTT1 in does-dependent manner in human granulosa-like tumor cells (KGN cells)[30]. Therefore, I examined whether the expression of GSTs and Nrf2, a transcription factor for GSTs, were induced by FSH stimuli in the same manner as GSTT1. FSH induced the expression of FSH receptor and GSTT1. In contrast GSTP1, GSTA1, GSTM and Nrf2 were not induced by FSH (Fig. 2-2). Likewise, immunofluorescence study revealed that GSTT1 protein was significantly induced by FSH stimuli in KGN cells unlike GSTP and Nrf2 (Fig. 2-3).

Nrf2 inhibitor, all-trans retinoic acid (ATRA), suppress other GSTs, but not GSTT1 expression

To clarify the relation between GSTT1 and Nrf2 in detail I analyzed the effect of all-trans retinoic acid (ATRA), an Nrf2 inhibitor, which interferes with binding of Nrf2 to the ARE. KGN cells were treated with ATRA (1 μM) or DMSO for control for 24 h, and the mRNA expression levels of GSTs were examined by RT-PCR.

Expectedly GSTA1 and GSTM1 expression was significantly inhibited, and GSTP1 was also seemed to be down-regulated by ATRA. On the other hand, GSTT1 expression was not suppressed, and rather induced than inhibited by ATRA, though the significant difference was not observed (Fig. 2-4). Similar results were obtained from immunofluorescence studies (Fig. 2-5).

Expression of GSTT1 via p38 MAPK, but not via JNK

In a series of experiments, I predicted that GSTT1 might be expressed via different stress-signaling pathway from other GSTs. In general, p38 MAPK and JNK are known as major stress-signaling cascades and especially JNK is known to be inactivated by GSTP binding to JNK-complex molecules in various cell-types [27-29]. To examine whether GSTs expressions under oxidative-stress were affected by p38 MAPK and JNK inhibitors (SB203850 and SP600125, respectably), KGN cells were treated with H_2O_2 (300 μM) which was the optimal concentration to up-regulate GSTs expressions (Fig. 2-6), and with each inhibitor (10 μM) for 24 h. Subsequently, the expression levels of GSTT1, GSTP, Nrf2, and Bax were analyzed by immunofluorescence study. Bax is a major apoptosis marker for various cell types and involved in the signaling cascade of p38 MAPK. In previous study, Bax showed same

expression pattern as GSTT1 in the difference of COC maturity[30]. Predictably, GSTP and Nrf2 were suppressed by JNK inhibitor; on the other hand GSTT1 was not affected with it. In contrast, a significant suppression of GSTT1 was induced by p38 MAPK inhibitor likewise Bax (Fig. 2-6). These results indicate that GSTT1 is not mediated by JNK but involved p38 MAPK unlike other GSTs,

Discussion

Serum FSH is elevated toward the menopause and rising serum FSH has been believed to accelerate reproductive aging[66]. Ito et al. reported that p38 MAPK was activated by FSH in KGN cells[67], although it is unclear whether activation of p38 MAPK via FSH is implicated in reproductive senescence or in reproductive physiology, such as steroidogenesis in case with GSTT1. FSH is thought to act as an antiapoptotic molecule in ovaries and granulosa cells by increasing glutathione[68,69]. Furthermore, a p38 MAPK inhibitor was shown to promote the production of steroidogenic acute regulatory protein by FSH and to reduce the expression of P450 aromatase in rat granulosa cells[70], indicating that GSTT1 and p38 MAPK would affect this kind of signaling. Notably, hCG did not increase GSTT1 expression at any of the concentrations examined, although it has also been reported to possess an antiapoptotic activity[71]. Up-regulation of GSTT1 might be regarded as a self-defense response downstream of FSH signaling.

Retinoids such as retinoic acid (RA) are chemopreventive and chemotherapeutic agents. One source of RA is vitamin A, derived from dietary β-carotene. RA regulates cell proliferation, differentiation, and morphogenesis. It inhibits tumorigenesis through suppression of cell growth and stimulation of cellular

differentiation[72]. RA also promotes apoptosis, indicating that this property may contribute to its antitumor properties. It was conclude that this might be due in part to the expression of the bcl-2 proto-oncogenes after treatment with RA in PCC7-Mz1 embryonic carcinoma cells[73,74]. Hoang Thanh Chi, *et al* reported that ATRA-induced apoptosis in gastrointestinal stromal tumor (GIST-T1) cells was accompanied by the down-regulated expression of survivin and up-regulated expression of Bax protein. Moreover, ATRA suppressed the activity of KIT protein in GIST-T1 cells and its downstream signal, AKT activity, but not MAPK activity [75]. My results demonstrated GSTT1 expression was not suppressed by ATRA but seemed to be induced by it in KGN cells. It has been reported that RA induces the rapid activation of p38 MAPK and downstream MSK1[76-78]. Considering that GSTT1 expression was similar to Bax expression in human and mouse granulosa cells in previous study[30], GSTT1 expression might be activated by ATRA similar to p38 MAPK.

GSTP has been reported to be an inhibitor of JNK activity i*n vivo*[48]. It was noted that in nonstressed cells, the activity of JNK toward c-Jun was basal. Upon UV irradiation and H_2O_2 treatment, JNK activation and subsequent c-Jun phosphorylation were seen. These studies led to the purification and identification of GSTP as the inhibitor of JNK activity. In mouse hepatocytes, mGSTA4 was reported to

be strongly increased during oxidative stress possibly via JNK pathway and during proliferation via MEK/extracellular signal-regulated kinase pathway, and GSTA4 might be an endogenous regulator of JNK activity by direct binding [79]. In my result, GSTP was significantly inhibited by JNK inhibitor. On the other hand, GSTT1 expression was not affected by JNK inhibitor. Up-regulation of Nrf2 seems to be suppressed by both inhibitors. In fact, p38 MAPK signaling has been reported to be one of the activation signal of Nrf2[55]. However, Nrf2-ARE pathway is not necessary to the activation of p38 MAPK signaling[80]. The upstream signaling pathways involved in the induction of these phase II detoxification enzyme genes seems to be specific for inducer, gene and cell type[81]. I also conducted the same experiment by using NIH 3T3 cells treated with H_2O_2 (1 mM), and the similar result was observed (Fig. 2-S1, S2). It is concluded that unlike other GSTs, GSTT1 seems to be affected by p38 MAPK signaling not via Nrf2 activation induced by H_2O_2 stress, and that is characteristic for GSTT1.

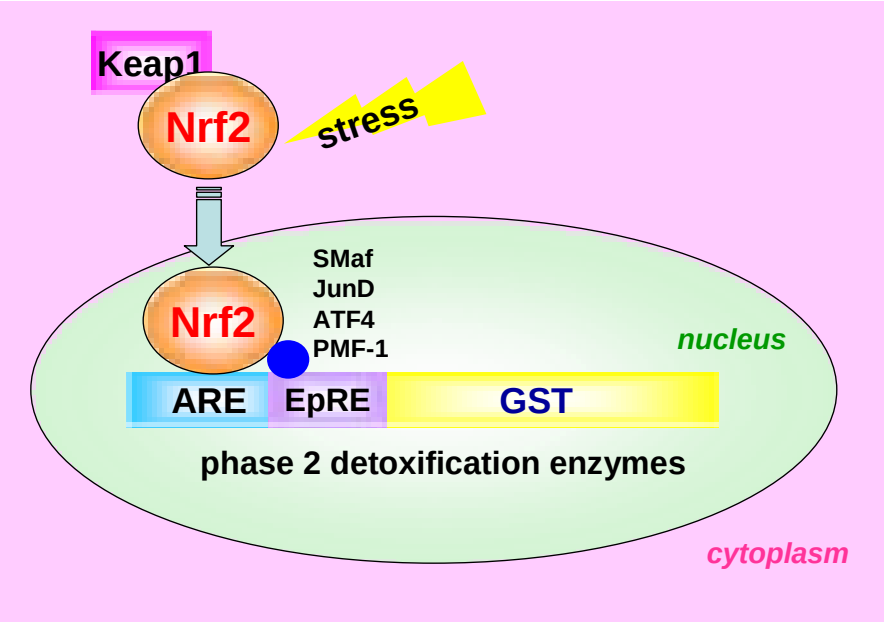


Fig. 2-1

Modulation of phase II detoxification enzyme genes expression through Nrf2/ARE pathway

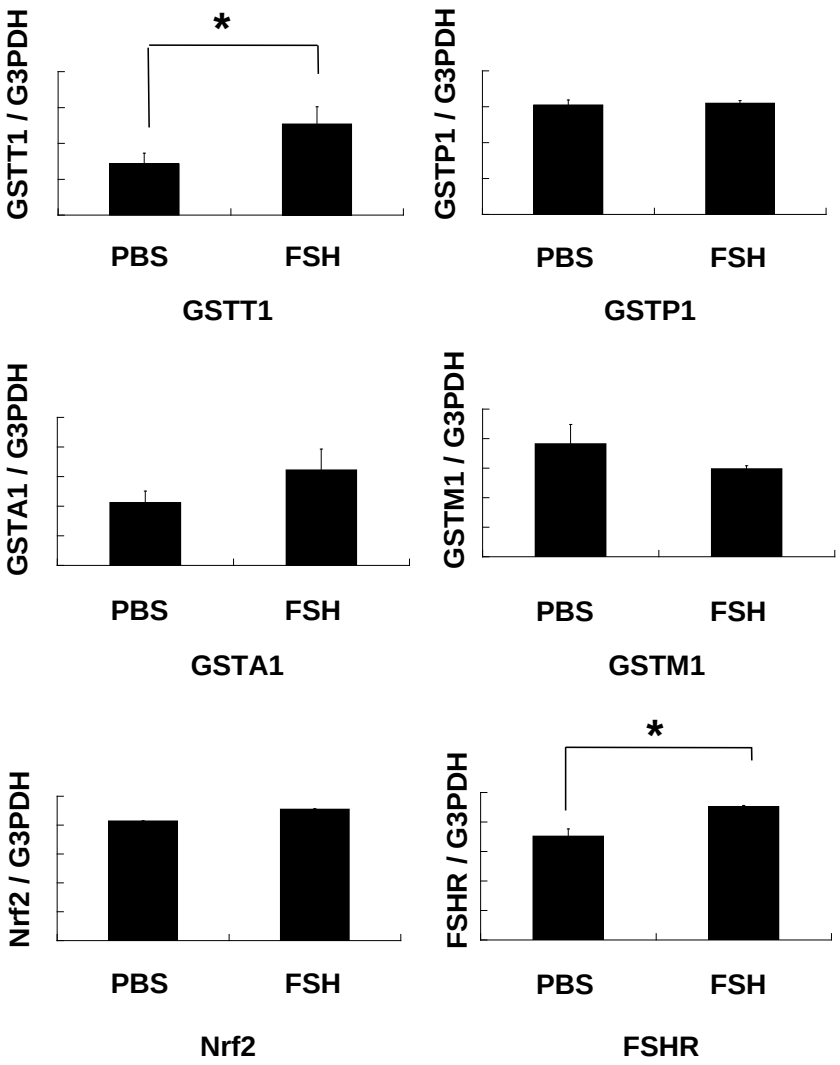


Fig. 2-2

The expression analysis of GSTs in FSH-treated KGN cells

Semi-quantitative RT-PCR analysis of GSTs in KGN cells treated with FSH (50 ng/ml) or PBS for control. The total RNA was isolated from theses cells, and RT-PCR analysis was performed using human specific primers for GSTs. Theses graphs represent the averaged band intensity of GSTs with SEM, normalized with G3PDH from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

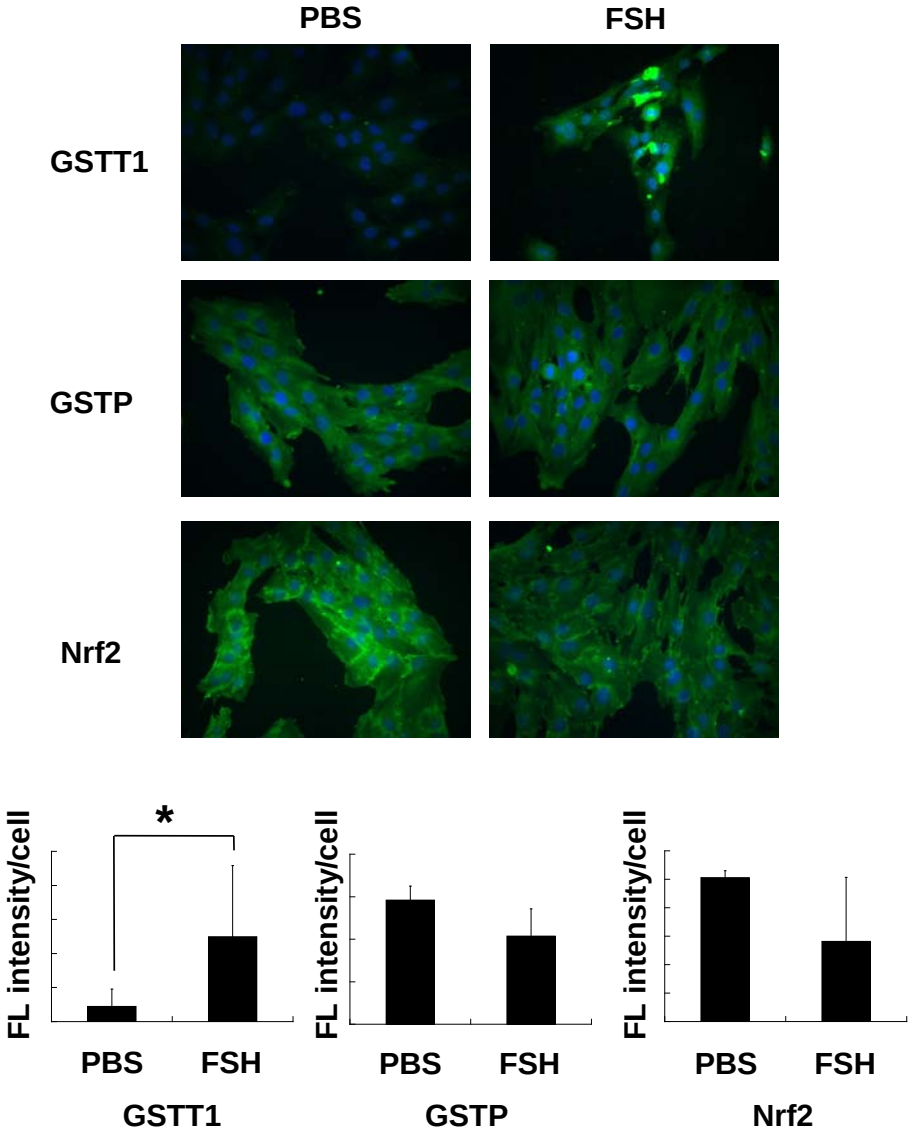


Fig. 2-3

The expression analysis of GSTs in FSH-treated KGN cells

The mean fluorescence intensity of GSTT1, GSTP and Nrf2 were measured in KGN cells treated with FSH (50 ng/ml) or PBS for control group and normalized with the number of cells. The upper panels show typical microphotographs from KGN cells treated with or without FSH. The cells were counterstained with Hoechst 33342 at 10 μM to visualize the nuclei (blue). The lower graphs show the mean fluorescence intensity of GSTT1, GSTP and Nrf2 per cell with SEM, and were analyzed by Student's or modified *t*-test (*P < 0.05).

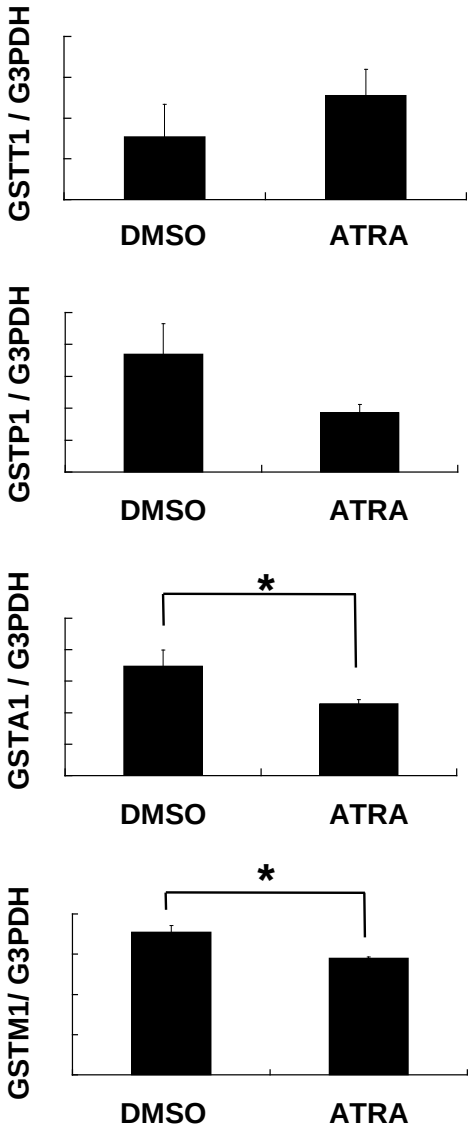


Fig. 2-4

The expression analysis of GSTs in KGN cells treated with Nrf2 inhibitor, all-trans retinoic acid (ATRA)

Semi-quantitative RT-PCR analysis of GSTs in KGN cells treated with ATRA (1 μM) or DMSO for control. The total RNA were isolated from theses cells, and RT-PCR analysis was performed using human specific primers for GSTs. Theses graphs represent the averaged band intensity of GSTs with SEM, normalized with G3PDH from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

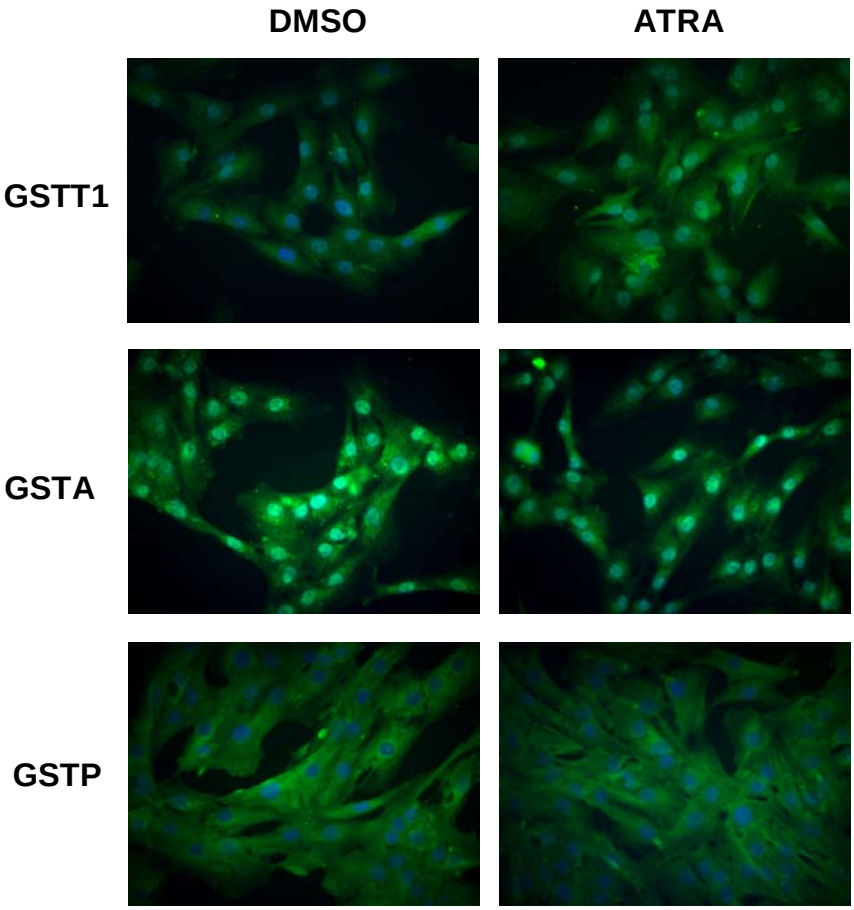


Fig. 2-5

The expression analysis of GSTs in KGN cells treated with Nrf2 inhibitor, all-trans retinoic acid (ATRA)

Immunostaining of GSTT1, GSTA and GSTP protein (green) in KGN cells treated with ATRA (1 μM) or DMSO for control. Theses panels show typical microphotographs from them. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue).

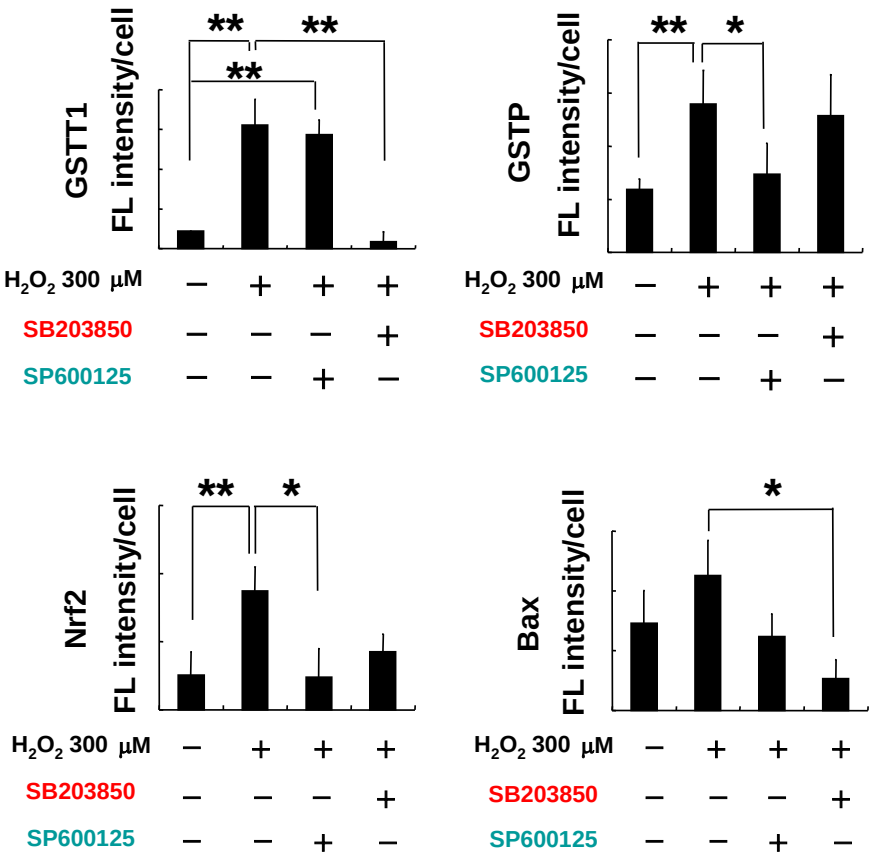


Fig.2-6

Immunofluorescence analysis of GSTs expression in H_2O_2-treated KGN cells with or without SB20385 or SP600125

KGN cells were treated with H_2O_2 at 300 μM for 24 h with or without SB20385 (10 μM) and SP600125 (10 μM). The integrated fluorescence intensity of GSTT1, GSTP, Nrf2 and Bax in each group (no stimuli, H_2O_2 300 μM, H_2O_2 300 μM+SB20385, H_2O_2 300 μM+SP600125) were 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 Student's *t*-test (*P < 0.05, **P < 0.01).

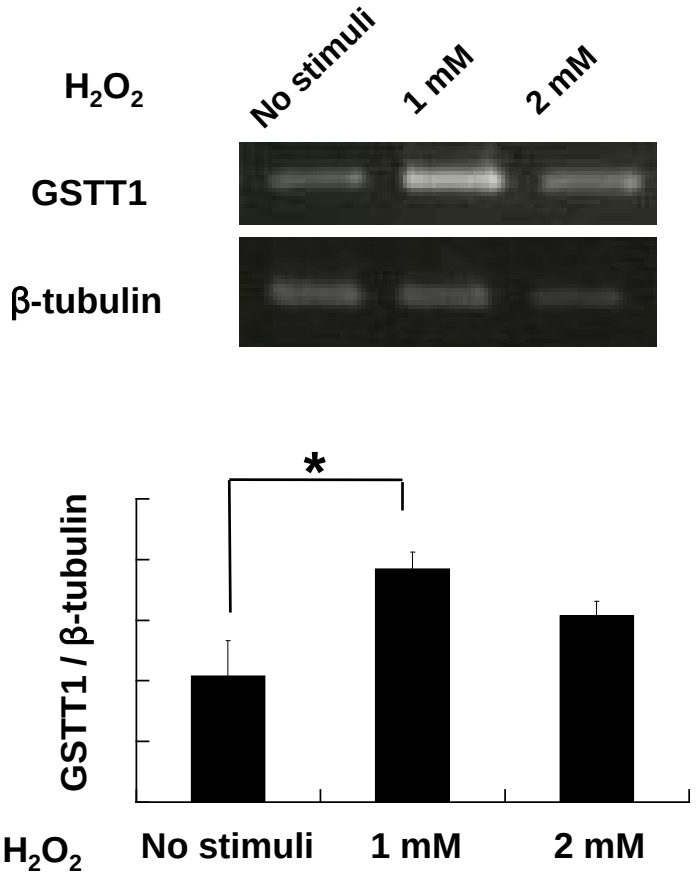


Fig. 2-S1

The expression analysis of GSTT1 in H_2O_2-treated NIH 3T3 cells

Semi-quantitative RT-PCR analysis of GSTT1 in NIH 3T3 cells treated with H_2O_2. The total RNA was isolated from theses cells, and RT-PCR analysis was performed using mouse specific primers for GSTT1.

Upper panel shows typical band of mRNA expression of GSTT1 in treated with H_2O_2 (1 mM and 2 mM) and lower graph represents the averaged band intensity of GSTT1 in these cells with SEM, normalized with β-tubulin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*$P < 0.05$).

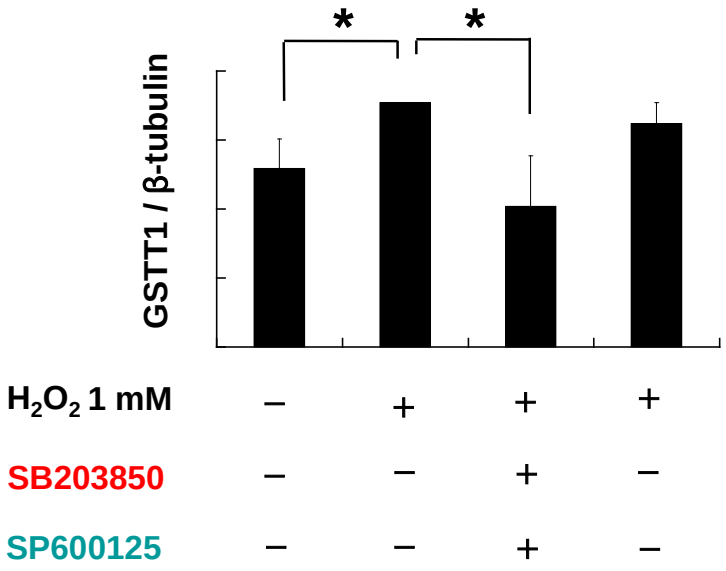


Fig. 2-S2

The expression analysis of GSTT1 in H_2O_2-treated NIH 3T3 cells with or without p38 MAPK and JNK inhibitor

Semi-quantitative RT-PCR analysis of GSTT1 in NIH 3T3 cells treated with H_2O_2 at 1mM and with or without SB20385 (10 μM) and SP600125 (10 μM). The total RNA was isolated from theses cells, and RT-PCR analysis was performed using mouse specific primers for GSTT1. The graph represents the averaged band intensity of GSTT1 in these cells with SEM, normalized with β-tubulin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

Chapter 3

Mechanism of transcriptional regulation of GSTT1

Abstract

GSTs are classified as phase II detoxification enzymes and known to be regulated by Nrf2, a transcription factor in general. Nrf2 is a DNA-binding protein for antioxidant response element (ARE) of the promoter regions and activated by oxidative stress and electrophiles, regulating the expression of numerous detoxification and antioxidant genes including GSTs. Previous studies indicated that GSTT1 expression was different from the other GSTs and seemed not to be regulated by Nrf2. In this chapter, I examined the putative Nrf2 binding site of GSTT1, compared with those of other GST genes, and found out several mutations of this element of GSTT1. Also, we suggested that GSTT1 was an aging marker for granulosa cells, and revealed that this phenomenon was true of not only reproductive cells, but also somatic cells. GSTT1 expression turned out to be up-regulated by aging especially in kidney. Next, I conducted over-expression analysis as well as siRNA silencing of GSTT1 using NIH 3T3 cells and MH1 cells. Then I focused on GATA factors of transcription factor, which operate downstream of p38 MAPK and involved in biological responses via various hormones including FSH. As a result, GATA-1 and GATA-6 expression were significantly up-regulated in NIH 3T3 cells with over-expression of GSTT1. On the other hand, GATA-4 was up-regulated in NIH 3T3 cells or MH-1 cells with siRNA

silencing of GSTT1. Interestingly, the over-expression of GATA-1 induced GSTT1 expression in NIH 3T3 cells and HM-1 cells. These results indicate that GSTT1 expression may be differently regulated from other GSTs, and mediated by p38 MAPK cascade via GATA factors. Especially, the transcription factor GATA-1 might be involved in the induction of GSTT1 expression.

Introduction

GSTs are classified as phase II detoxification enzymes and known to be regulated by nuclear factor erythroid 2 p45-related factor 2 (Nrf2), a transcription factor[31,32]. Most of these protective genes have a common cis-element, the antioxidant response element (ARE) in their promoter regions for which Nrf2 binds after stimulation by oxidative stress and electrophiles, and regulates the expression of numerous detoxification and antioxidant genes including GSTs[33,34,82-86]. Keap1, the partner of Nrf2, directly interacts with Nrf2, and transmit to ARE by the inducer signal. Keap1 also plays an active role in Nrf2 regulation, by directing it for proteasomal degradation. Upon induction, Nrf2 is believed to dissociate from the Keap1-Nrf2 complex and translocate into the nucleus and bind ARE to enhance the expression of phase II detoxification enzyme genes[44,87,88], although the exact mechanism for the interaction between inducers and Keap1 (or Nrf2) is still unclear.

Induction of phase II detoxification enzyme gene has been implicated in a variety of upstream signaling pathways, one of which is p38 MAPK pathway. p38 MAPK plays a pivotal role in multiple physiological activities, and activates various transcription factors including ATF-1/2, Sap1, GADD153, GATA factors, and p53[89-96]. Among them, GATA factors, GATA-1 to GATA-6, are a family of zinc

finger transcription factors that bind to a consensus DNA sequence (A/T)GATA(A/G) in gene promoters and enhancers[97]. GATA-1 to GATA-3 are expressed principally in hematopoietic cells, whereas GATA-4 to GATA-6 regulate gene expression, differentiation, cellular proliferation, and apoptosis in various mesoderm- and endoderm-derived tissues including heart, gut, gonads, and adrenal cortex[98,99]. GATA-4 and GATA-6 are expressed in the adult endocrine tissues including testes and ovaries[100,101]. The maturing follicles of the adult ovary exclusively express GATA-4 and GATA-6, regulating genes important to ovarian function including several genes mediating the production of steroids[100], but few other ovarian GATA targets are known. Pregnant mare serum gonadotropin (PMSG) enhances follicular expression of GATA-4 and GATA-6 transcripts in immature mice (3 weeks)[102]. GATA-4 is induced by FSH and involved in protein kinase A and p38 MAPK pathway activated by gonadotropins and cAMP (Fig.3-1)[102,103], whereas in mature human granulosa cells from the patients undergoing infertility treatment, FSH was shown to up-regulate GATA-6 mRNA[104]. In the mouse ovary GATA-4 is expressed in granulosa cells of primary, secondary, and antral follicles. GATA-4 transcripts are detected in lesser amounts in theca cells and interstitial cells. In contrast, GATA-6 messenger RNA (mRNA) is expressed only in granulosa cells of late antral follicles, preovulatory

follicles, and in corpus luteum[102]. The effects on GATA-6 rather than GATA-4 likely reflect the luteinzed phenotype of human granulosa cells. GATA-1 also exhibit gonadal expression especially in Sertoli cells and Leydig cells[105], indicating the association with FSH. In addition, it has been shown that rat FSH receptor promoter possesses a functional GATA-1 site[106].

In my previous studies, GSTT1 expression was induced by age-related stress and FSH in granulosa cells, and this phenomenon may be different from other GSTs. Therefore, first I examined the expression pattern of GSTT1 in other tissues, including liver and kidney. Next the association between GSTT1 and GATA factors was investigated by the over-expression analysis as well as siRNA silencing of GSTT1, using NIH 3T3 and HM-1 cells, a mouse ovarian tumor cell line.

Materials and Methods

Reagents

Mouse monoclonal anti-FLAG M2 antibody was purchased from Sigma Chemical Co (St. Louis, MO). Rabbit polyclonal antibodies against GATA-4 and GATA-1 were purchased from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA) and abcam (Tokyo, Japan), respectively. Rabbit polyclonal antibody against phosphorylated p38 (pp38) was purchased from Sigma Chemical Co (St. Louis, MO, USA). The anti-V5 FITC antibody was purchased from Invitrogen Life Technologies (Tokyo, Japan).

Cell culture and preparation

NIH 3T3 cells have been maintained in our laboratory. The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from the RIKEN BioResource Center (Tsukuba, Japan). NIH 3T3 cells were maintained in a Dulbecco's modified Eagle's medium (DMEM, Sigma) and HM-1 cells were maintained in a MEM alpha medium (Invitrogen) at 37°C in a CO_2 incubator, both supplemented with 10% heat-inactivated fetal calf serum, 100 μg/ml penicillin and 100 IU/ml streptomycin. Cells were seeded and cultured into 6 cm dishes or 8-well culture

slides (BD Japan Co. Ltd., Tokyo, Japan) for transfections.

Plasmid construction and transfection

The vectors for mouse GSTT1 and GATA-1 over-expression were created. Briefly, mouse GSTT1 gene added the restriction enzyme cleavage site without stop codon was amplified by the polymerase chain reaction using the specific primers and inserted into pCMV-Tag1 epitope tagging mammalian expression vector (Agilent Technologies, North Torrey Pines Road La Jolla, CA). The vector was digested with the restriction enzyme and inserted into pCAGGS vector (kindly provided by Dr. J. Miyazaki, Osaka University) as described (Fig.3-2 pCAGGS-GSTT1-FLAG). The pLenti6/GATA-1-V5 TOPO vector (Invitrogen, Tokyo, Japan) encoding mouse GATA-1 was prepared according to the manufacturer's instruction (Fig.3-3). NIH 3T3 cells and HM-1 cells were grown to 50% confluent for transfection. Transient transfections were carried out using FuGENE HD transfection reagent (Promega, L.L.C. USA) according to the manufacturer's instruction. The medium was changed to fresh one at 24 h after transfection. After 24 h incubation, the cells cultured in 6 cm dishes were transferred into 1.5 mL microtubes, centrifuged for 5 min at 15,000 rpm at 4°C to remove excess buffer, and stored at –80°C until use. The cells cultured in 8-well culture slides were fixed in 4% paraformaldehyde in PBS + BSA for 15 min at room temperature, washed

three times in PBS + BSA, put on glass slides, air-dried, and then stored at 4°C until use.

Small interfering RNA

siRNA-mediated knockdown for mouse GSTT1 was performed in NIH 3T3 and HM-1 cells. The piLentiviral vector for siRNA-mediated knock down of mouse GSTT1 was constructed with piLenti siRNA-GFP vector (Funakoshi, Tokyo, Japan) encoding shRNA target sequence: 5'-GGCACACAAGTATAAGGTTCCCGAAGGAACCTTATACTTGTGTGCC3-', and pENTR/U6 vector (Invitrogen, Tokyo, Japan) encoding: 5'-CACCGGCACACAAGTA TAAGGTTCCCGAAGGAACCTTATACTTGTGTGCC-3'. The map and structure of these vectors are shown in Fig3-4. The cells were transfected as described above.

Immunofluorescence examination

The immunofluorescence staining method of NIH 3T3, HM-1 and, KGN cells were described previously. The rabbit polyclonal antibody against GATA-1 was diluted at 5 μg/ml, goat polyclonal antibody against GATA-4 was diluted at 10 μg/ml, and rabbit polyclonal antibody against pp38 was diluted at 20 μg/ml and incubated with these samples for overnight at 4°C, and visualized with goat anti-rabbit IgG conjugated

with Alexa Fluor 488 and donkey anti-goat IgG conjugated with Alexa Fluor 488 diluted at 1:1,000 in PBS + 0.1% Triton X-100 for 1 h at room temperature, respectively. The anti-V5 FITC antibody was diluted at 1:5,000 in PBS + 0.1% Triton X-100 for the detection of V5 and all experiments were carried out by the same procedure as described in Chapter 1.

Western blot analysis

The transfected cells were suspended in PRO-PREP protein extraction solution buffer (iNtRON Biotechnology, Inc., Chunmin-Dong, KOREA) with a protease inhibitor cocktail (Roche), and incubated at 4°C for 30 min. Then the cells were centrifuged for 5 min at 15,000 rpm at 4°C to remove insoluble material. The supernatants were loaded in protein sample loading buffer, and run on 4–20% mini-protean TGX gels (Bio-Rad Laboratories, Inc., Tokyo, Japan) and then transferred onto a PVDF membrane (Amersham Hybond-P; GE Healthcare, Piscataway, NJ, USA). After blocking with 100% Block Ace (Snow Brand Milk Products Co., Tokyo, Japan) at room temperature, membranes were processed through sequential incubations with anti-FLAG M2 antibody diluted at 1:1000 in 1% BSA/TBS or anti-beta actin monoclonal antibody diluted at 1:1,500 in 1% BSA/TBS for 1 h and then with

horseradish peroxidase-conjugated anti-mouse IgG.. ELC Prime Western Biotting Detection Reagent (GE Healthcare, Piscataway, NJ, USA) was then used for visualization, and the signal was developed on an X-ray film (Amersham Hyperfilm ECL; GE Healthcare, Piscataway, NJ, USA).

Semi-quantitative RT-PCR analysis

Reverse transcription using total RNA isolated from the livers and kidneys from young (8 weeks-old) and aged (50 weeks-old) female C57BL/6J mice and NIH 3T3 cells were performed as described in Chapter 1. The expressions of mRNA were then monitored by RT-PCR analysis using the primer sets described in Chapter 1 (Table 1-1).

Statistical analysis

Normalized data on the mRNA expressions are shown as the means with standard error of the means (SEM) from at least three independent experiments. These data were analyzed with Student's *t*-test or modified *t*-test (Welch's correction). Differences were considered statistically significant when $P<0.05$. These analyses were performed using Microsoft Excel software.

Results

GST theta class had several mutations in the putative Nrf2 binding antioxidant response element (ARE) in their promoter regions

Phase II detoxification enzymes are known to be regulated by Nrf2 and have the antioxidant response element (ARE) in their promoter regions, which is putative Nrf2 binding site[31,32]. However, our previous study indicated that GSTT1 seemed not to be regulated by Nrf2. To understand the relationship between Nrf2 and GSTT1 expression, the sequence for the putative Nrf2 binding sites of GSTs, the upstream of 2.0 kb was explored computationally by using Molecular Genetics Explorer (MGX). In mouse, theta class has four members, GSTT1, GSTT2, GSTT3 and GSTT4. Table 3-1 shows the putative Nrf2 binding sites of mouse GST genes. The ARE sequence (TMAnn RTGAYnnn GCR WWWW) was conserved in Human, Mouse and Rat[107]. Quinone reductase (GR) [NAD(P)H:(quinone acceptor) oxidoreductase, EC 1.6.99.2], also called DT diaphorase, is also classified as phase II detoxification enzymes and is a homodimeric FAD-containing enzyme that catalyzes obligatory NAD(P)H-dependent two-electron reductions of quinones and protects cells against the toxic and neoplastic effects of free radicals and reactive oxygen species arising from one-electron reductions[72,108,109]. QR is known to be regulated by Nrf2-ARE

pathway, and ARE core sequences is identified in the promoter regions of QR genes in human and rat (Table 3-1). Also, GSTA1, GSTP1 and GSTM1 contain ARE sequences, previously reported[107]. However, genes of GST theta class in both human and mouse possessed some mutations in the ARE consensus sequence. To conclude, it is implicated that GSTT1 is not regulated by Nrf2 in contrast to other GSTs.

Up-regulation of GSTT1 expression in the kidney from aged mice

In our previous study, GSTT1 was up-regulated in aged-granulosa cells from both human and mouse. In this study I examined the expression patterns of GSTT1 in the liver and kidney from young (8 week-old) and aged (50 week-old) mice, because the detoxification enzymes including GSTs are highly expressed in these organs. GSTP1 mRNA level in the livers from young mice was significantly higher than that from old mice. However, there was no significant difference in the mRNA level in the kidney. On the other hand, GSTT1 mRNA was up-regulated in aged kidneys (Fig. 3-5). Age-dependent difference in Nrf2 mRNA level was not observed in the liver and kidney (Fig. 3-5). These data indicate that age-dependent high expression of GSTT1 was observed not only in granulosa cells, but also in some somatic cells.

Evidence that GSTT1 expression may be mediated by GATA factors

A series of experiments suggest that GSTT1 may not be regulated by Nrf2-ARE pathway, and implicated in p38 MAPK signaling cascade. To explore what is involved in the regulation of GSTT1, especially the downstream of p38 MAPK, I conducted overexpression analysis as well as siRNA silencing in NIH 3T3 cells. The cells were transfected with pCAGGS-GSTT1-Flag vector described in Fig.3-2. The efficacy of GSTT1 overexpression was checked by Western blot and immunofluorescence analyses. FLAG expression in the cells transfected with pCAGGS-GSTT1-Flag vector was verified, and enhanced GSTT1 expression was confirmed in these cells, compared to control (Fig. 3-6). NIH3T3 cells were transfected with piLenti siRNA GSTT1-GFP vector described in Fig.3-4. The transfection efficacy of GSTT1 siRNA was checked by RT-PCR for GFP. GFP in the cells transfected with piLenti-GFP-GSTT1 was detected under a fluorescence microscope (Fig. 3-7). Gene expression in the GSTT1-overexpressed or siRNA mediated GSTT1-downregulated cells was examined by RT-PCR analysis. A significant increase and decrease of GSTT1 mRNA expression were observed in GSTT1 over-expressed and siRNA-mediated GSTT1-downregulated NIH 3T3 cells, respectively. GSTP1 mRNA level was not changed in these cells (Fig. 3-8). mRNA level of Bcl2, an antiapoptotic family member,

and Bax, an apoptosis marker were examined in these cells. Although Bcl-2 mRNA level stayed unchanged, Bax mRNA expression was significantly up-regulated in the GSTT1-overexpressed cells. Interestingly, the downstream of p38 MAPK, GATA factors showed different expression patterns. The overexpression of GSTT1 increased GATA-6 mRNA expression, while siRNA-mediated down-regulation of GSTT1 increased GATA-4 mRNA level and down-regulated GATA-1 mRNA expression (Fig. 3-8). Similar experiments were conducted using HM-1 cells, mouse ovarian tumor cell line, which is very sensitive to hormones. HM-1 cells were transfected with pENTR/U6-GSTT1 vector, and siRNA mediated knockdown of GSTT1 was preformed. Decreased expression of GSTT1 protein was determined in immunofluorescence analysis. Also, GATA-1 protein was down-regulated, while GATA-4 protein was up-regulated in HM-1 cells with GSTT1 siRNA (Fig. 3-9). These results suggest that induction of GSTT1 expression could be directly or indirectly mediated by GATA factors in epithelial cells.

GATA-1 is one of the regulators of GSTT1 expression in NIH 3T3 cells

Base on the above results, I examined the relationship between GSTT1 and GATA-1 expression. The microarray analysis showed that GSTT1 expression in

megakaryocytes lacking GATA-1 obtained from 13.5 days mutant embryos of C57BL/6 mice is significantly decreased[110], predicting that GATA-1 might be regulate GSTT1 expression. Then, overexpression of GATA-1 was performed and gene expression was examined in NIH 3T3 cells. The cells were transfected with pLenti6/V5 TOPO vector encoding GATA-1 gene sequence (Fig. 3-3 pLenti6/GATA-1-V5). The efficacy of overexpression of GATA-1 was verified by immunofluorescence analysis, and high expression of GATA-1 and V5 was observed in the nucleus of cells overexpressed with GATA-1 (Fig. 3-10). GSTT1 expression was also up-regulated in these cells (Fig. 3-10, 11). Gene expression in the GATA-1-overexpressed NIH 3T3 cells was examined by RT-PCR. Interestingly, GSTP1 and GSTA1 mRNA levels were down-regulated, contrary to GSTT1 expression (Fig. 3-11). Also, Bax expression was significantly up-regulated with induction of GSTT1. In addition, I examined whether or not FSH induced the expressions of GATA-1 and p38 in KGN cells in the same manner as GSTT1. Expectedly, immunofluorescence study revealed that GATA-1 and pp38 expressions were up-regulated in FSH-treated KGN cells (Fig. 3-12).

Discussion

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[111]. GSTs can also inhibit Jun N-terminal kinase, resulting in protection of cells against H_2O_2-induced cell death [112]. However GSTT1 expression was not suppressed by JNK inhibitor, unlike GSTP1 in KGN cells (Fig.2-6). Interestingly, GSTT1 could not bind to GSH-agarose or S-hexyl-GSH-agarose, unlike other GSTs [113]. GSTT1 has been reported to have conflicting properties, showing detoxification activity and production of a genotoxic metabolite [114]. Taken together, the mechanism of expression of GSTT1 might be completely different from that of other GSTs.

In this study, I explored the putative Nrf-2 binding site of human and mouse GSTT1 genes. Favreau and Pickett reported that essential ARE sequence was RTGACnnnGCA and mutations in G-C dinucleotide reduced both constitutive and induced expression in the mutagenesis study of the rat QR ARE [86]. Also, this tandem core mutation in human QR ARE decreased induction by 80% compared with the wild-type ARE[115]. Compared to other phase II detoxification enzymes, several mutations were identified in the ARE tandem core of GST theta class, predicting that

GSTT1 may not be regulated by Nrf2-ARE pathway.

We previously revealed that GSTT1 expression was induced by the stress increased by aging, for example oxidative stress and FSH in human granulosa cells[30]. In this study I found that not only in granulosa cells, but also in renal cells, GSTT1 expression was up-regulated in aged mice. From the profiling study on the expression of senescence-associated genes in biopsies of dermis from young and old human donors, glutathione peroxidase and GSTT1 were up-regulated in old dermis, and the authors concluded that GSTT1 protected against oxidative stress generated by UV irradiation[116]. They suggest that GSTT1 up-regulation could be due to the concentration of ROS increased with aging and be one of antioxidant defense system in epidarmal cells [116]. Likewise, up-regulation of GSTT1 in both granulosa and renal cells could respond to age-related stress and protect cells from them in a similar way to other detoxification enzymes.

However, GSTT1 overexpression study showed subsequent up-regulation of Bax expression. We previously demonstrated that the expression patterns of GSTT1 and Bax were comparable in human cumulus-oocyte complex (COC) sorted according to the maturity[30]. Furthermore, both GSTT1 and Bax were expressed very strongly in mouse atretic follicles, indicating that GSTT1 was an indicator for the apoptosis of the

granulosa cells. In fact, the study using V79 cell line stably transfected with murine GST theta 1 gene demonstrated that GSTT1 could induce DNA damage and cytotoxicity through the formation of DNA–protein crosslinks in the metabolic process of dichloromethane, which is catalyzed by only GSTT1[117]. In this context, even though GSTT1 may not have apoptotic function, the metabolites of GSTT1 could induce apoptotic molecules such as Bax. In my preliminary experiment, however, significant difference in the cell ability was not observed in mouse oocytes with over-expression of GSTT1 (data not shown). The biological significance of this finding remains obscure.

Unexpectedly, GATA-1 induction increased GSTT1 expression. GATA-1 is expressed in gonadal cells including Sertoli cells and Leydig cells[105], and also expressed in hematopoietic cells. GATA-1 is required for the development of megakaryocytes and erythroid cells and believed to be a key regulator of erythropoiesis[98]. In addition, there is an evidence that GSTT1 expression in megakaryocytes lacking GATA-1 obtained from 13.5 days mutant embryos of C57BL/6 mice is significantly decreased[110].

A member of the sucrose nonfermenting protein kinase (SNF1)/AMPK serine/threonine kinase family, Melk was initially cloned in mouse oocytes as a maternal gene, and in adult mice, strongly expressed in hematopoietic organs such as

the thymus and spleen. This family is thought to play a role in protecting cells from environmental stress, hypoxia, heat shock, and ischemia[118,119]. It was reported that expression of Melk-like gene in zebra fish embryos enhanced GATA-1 promoter-dependent expression, suggesting that Melk-like gene affects GATA-1 expression at the transcriptional level[120]. Self-defense system in both Melk and GSTT1 is highly conserved among all organisms including prokaryotes and eukaryotes[111]. Considering that GSTT1 seems to have similar characteristic as Melk, GSTT1 expression could be regulated by GATA-1 like Melk expression. Up-regulation of GATA-1 was determined in FSH-treated KGN cells and also pp38 expression was increased in these cells. However, GATA-1 expression is scarcely expressed in ovarian cells differently from testicular cells[105]; it is vague whether GSTT1 is induced by GATA-1 in granulosa cells with aging. To find out this precise mechanism, a tissue-specific overexpression and down-regulation of GATA-1 study is needed. In this study, I demonstrated that GSTT1 might not be induced by Nrf2-ARE mediated pathway, and more likely to be associated with GATA factors, the downstream of p38 MAPK in some type of cells. GSTT1 may have different stress-response and stress-signaling cascades from other GSTs, and GSTT1 expression may be induced by GATA-1, contrary to other GSTs, and could be directly or indirectly interacted with Bax

and p38 MAPK pathway.

Table 3-1

ARE sequence, the putative Nrf2 binding sites, of phase II detoxification enzymes

gene	ARE consensus	TMAnn	RTGAYnnn	GCR	WWWW
Human QR		-TCAca	GTGACtcg	GCA	GAAT-
Rat QR		-TCAca	GTGACttg	GCA	AAAT-
Rat GSTA1		-TAAtg	GTGACaaa	GCA	ACTT-
Mouse GSTA1		-TAAtg	GTGACaaa	GCA	ACTT-
Mouse GSTP1		-TCAct	ATGATtca	GCA	ATAA-
Rat GSTP1		-TCAct	ATGATtca	GCA	ATAA-
Mouse GSTM1		-CTTcg	GTGACata	GCC	TCCA-
Human GSTT1		-TTAaa	CTCACttt	GCA	TTGG-
Mouse GSTT1		-TGGta	GTGACctg	GAG	GGGG-
Mouse GSTT2		-GCAgg	CTGAGcaa	GCC	AGGG-
Mouse GSTT3		-TAAgt	TTGAAaga	GCC	TTCC-
Mouse GSTT4		-TGGgt	GTGTCagt	GCT	GTGC-

M(A/C), R(A/G), Y(C/T), W(A/T), n(any nucleotide).

The mutation is shown as yellowed.

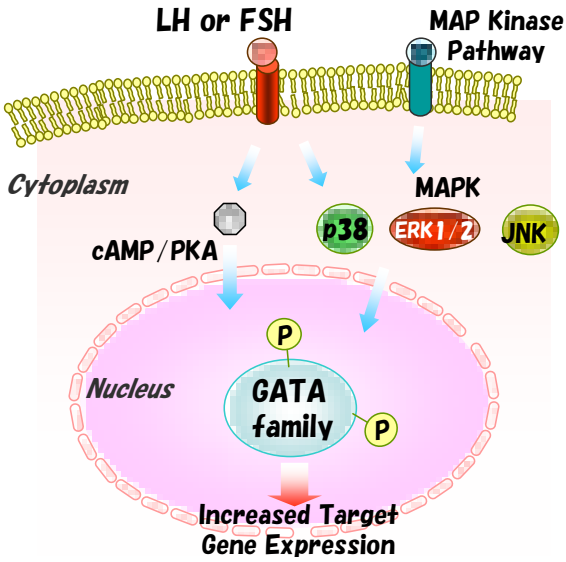


Fig. 3-1
Modulation of steroidogenic gene expression through phosphorylation of GATA factors

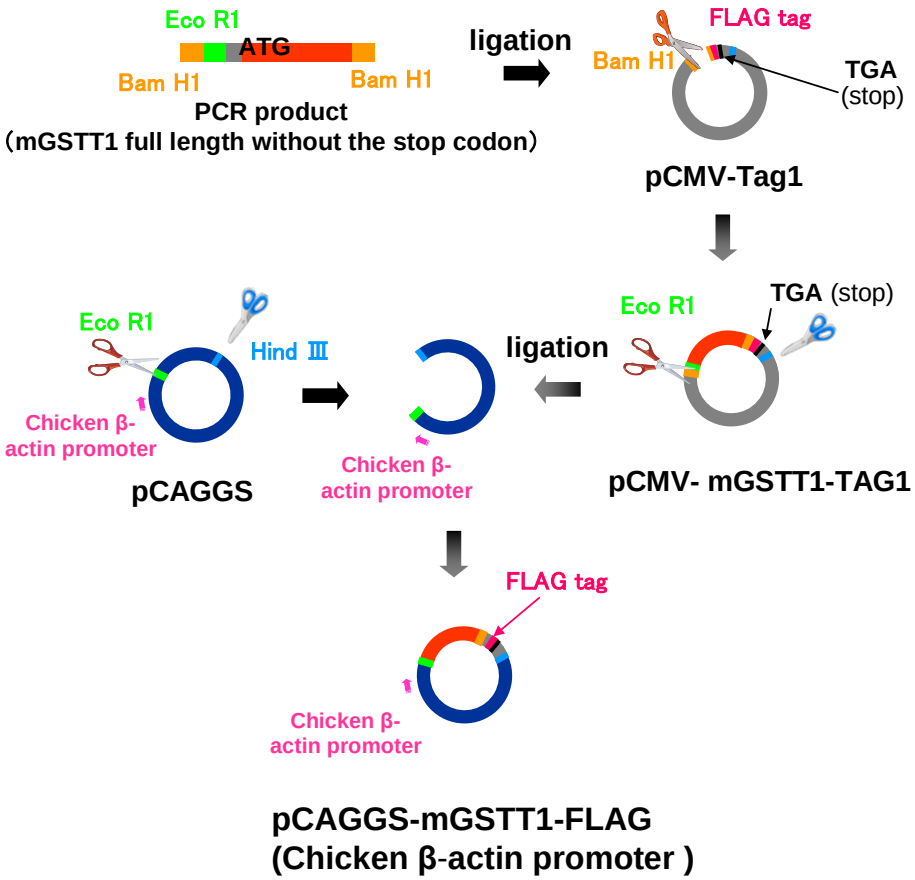
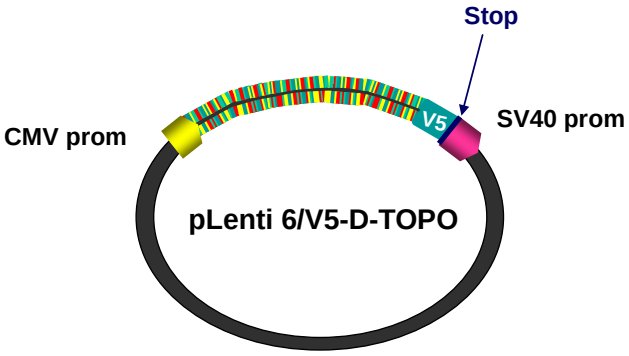


Fig. 3-2

Map and Features of pCAGGS-mGSTT1-FLAG for over-expression of GSTT1



pLenti6/GATA-1-V5

Fig. 3-3
Map and Features of piLenti 6/V5-D-TOPO for over-expression for mGATA-1

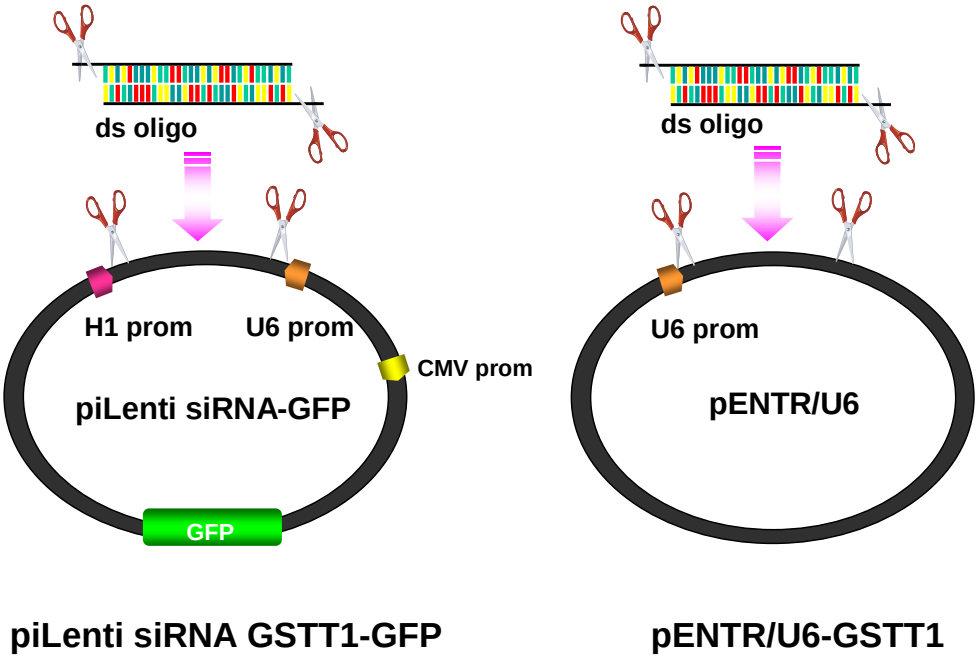


Fig. 3-4

Map and Features of piLenti siRNA-GFP and pENTR/U6 for suppressive-expression for mGSTT1

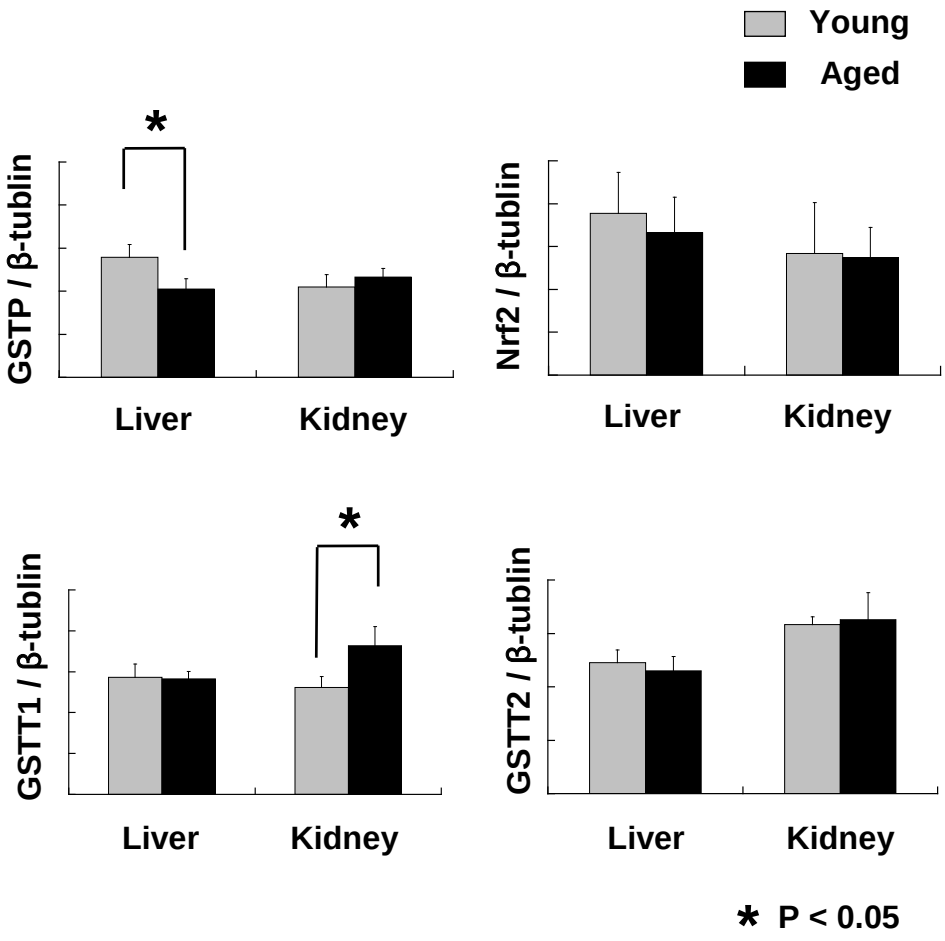


Fig. 3-5

GSTT1 expression in the liver and kidney of young and aged mice

Semi-quantitative RT-PCR analysis of GSTs in young (8 weeks-old) and aged (50 weeks-old) mice Total RNA were isolated from cumulus granulosa cells, and RT-PCR analysis was performed using mouse specific primers for GSTT1, GSTT2, GSTP1 and Nrf2. These graphs represent the averaged band intensity of GSTs with SEM, normalized with β-tubulin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

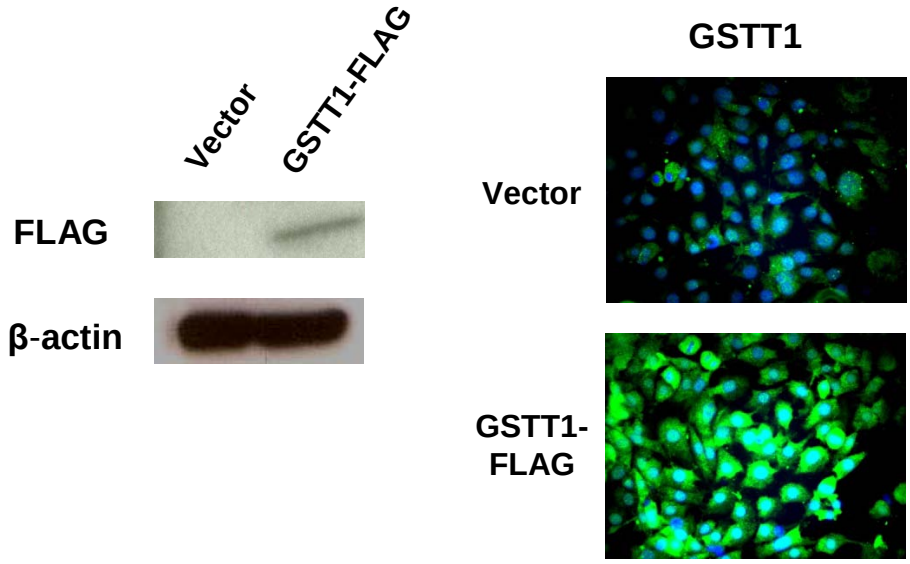


Fig. 3-6

Overexpression analysis of GSTT1 in NIH 3T3 cells

NIH 3T3 cells were transfected with overexpression vector encoding mGSTT1 and Flag tag (pCAGGS-GSTT1-Flag), and incubated for 24 h, and used for Western blot and immunofluorescence analyses.

Left panels present the expression of Flag and β-actin protein determined in those NIH 3T3 cells by Western blotting. Right panels show typical microphotographs of GSTT1 expression in these cells by immunofluorescence analysis.

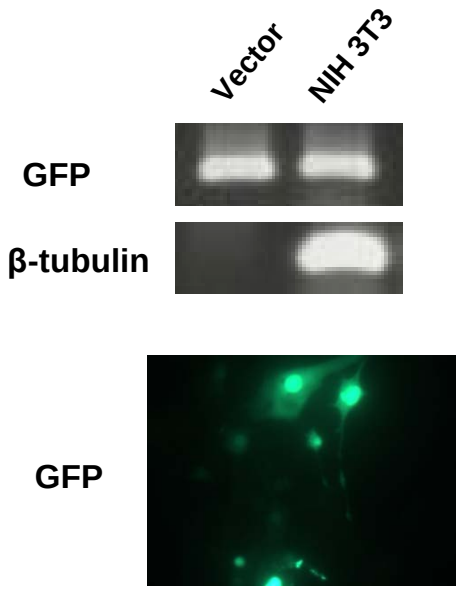


Fig. 3-7

GFP expression in NIH 3T3 cells transfected with piLenti siRNA GSTT1-GFP vector

NIH 3T3 cells were transfeccted with the lentiviral vector for siRNA mediated knockdown of mGSTT1 (iLenti siRNAGSTT1-GFP), and incubated for 24 h, and used for RT-PCR analysis.

Upper panel shows GFP mRNA expression. GFP protein expression in the transfected NIH 3T3 cells is shown in the lower panel.

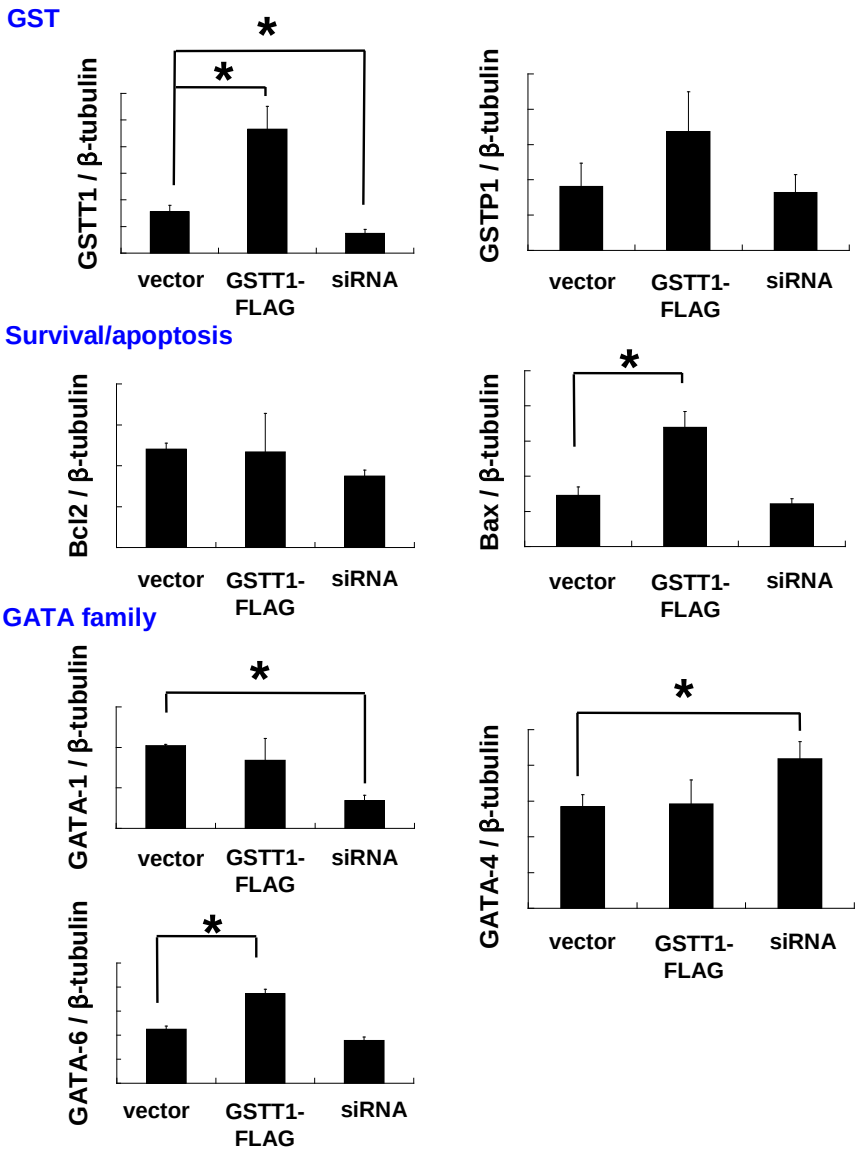


Fig. 3-8

Overexpression analysis and siRNA mediated knockdown of GSTT1 in NIH 3T3 cells

Semi-quantitative RT-PCR analysis of stress responsive genes in NIH 3T3 cells transfected with pCAGGS-GSTT1-Flag plasmid or iLenti siRNAGFP-GSTT1

The total RNA was isolated from those NIH 3T3 cells, and PCR analysis was performed using mouse specific primers for GSTs, Bcl2, Bax and GATA family. Theses graphs represent the averaged band intensity of those genes with SEM, normalized with β-tublin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (*P < 0.05).

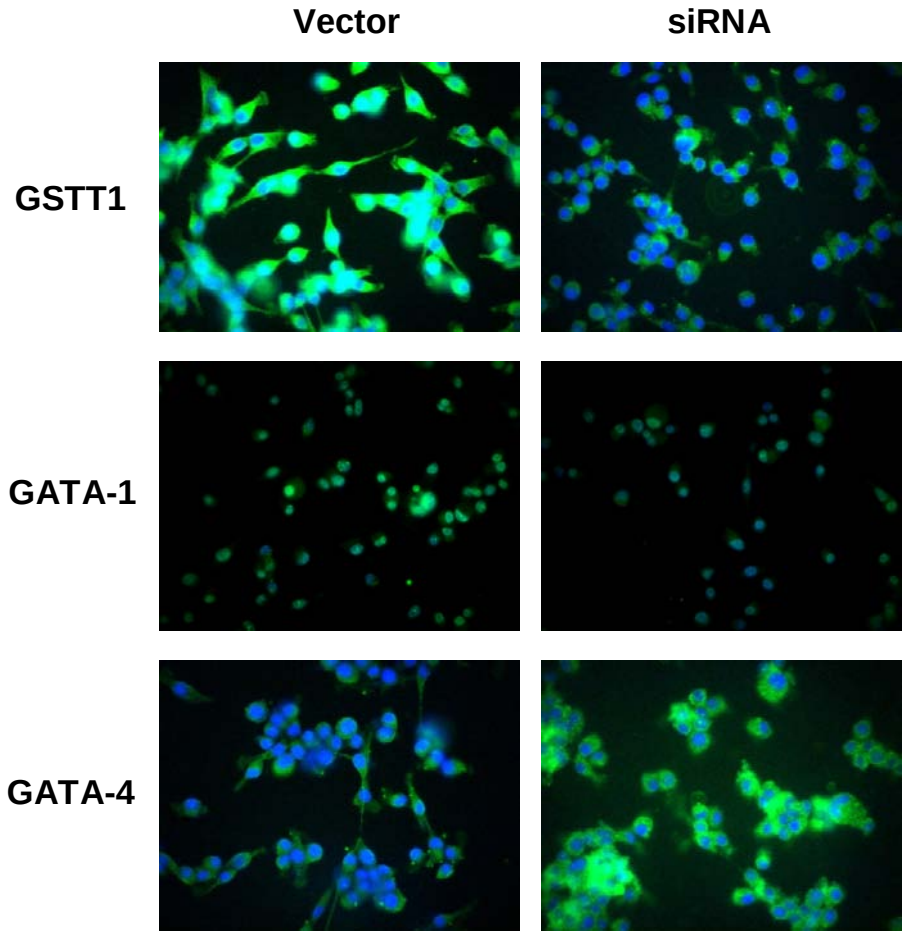


Fig. 3-9

siRNA mediated knockdown of GSTT1 in HM-1 cells

HM-1 cells were transfeccted with the vector for siRNA mediated knockdown of mGSTT1 (pENTR/U6-GSTT1), and incubated for 24 h, and used for immunofluorescence analyses. Immunostaining of GSTT1, GATA-1 and GATA-4 protein (green) in those HM-1 cells. Theses panels show typical microphotographs. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue).

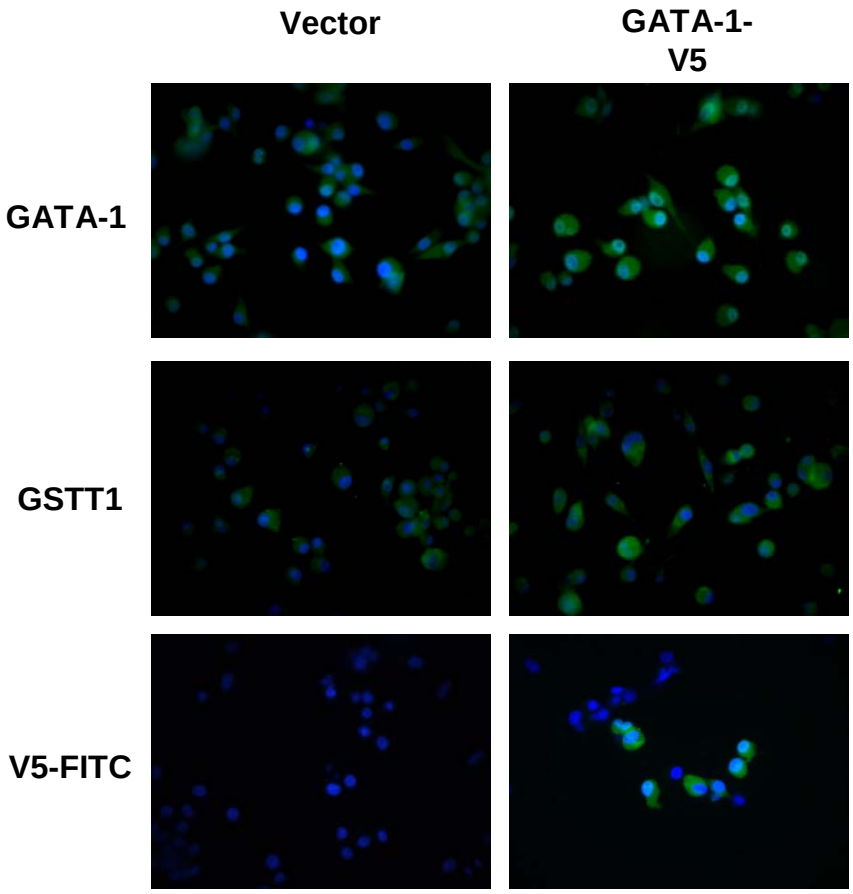


Fig. 3-10

Overexpression analysis of GATA-1 in NIH 3T3 cells

NIH 3T3 cells were transfected with overexpression vector encoding mGSTT1 (pLenti6/GATA-1-V5), and incubated for 24h, and used for immunofluorescence analyses. Immunostaining of GATA-1 and GSTT1 proteins and V5 tag (green) in those NIH 3T3 cells. These panels show typical microphotographs. The cells were counterstained with Hoechst 33342 to visualize the nuclei (blue).

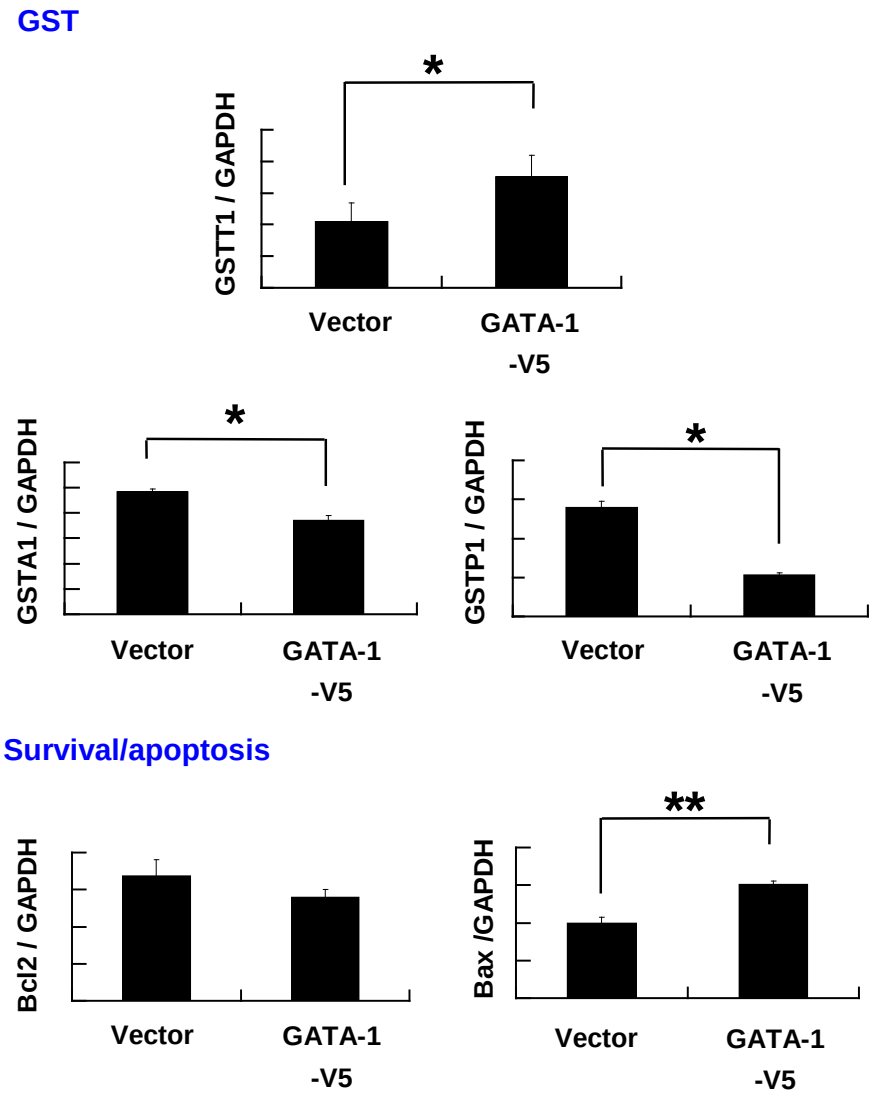


Fig. 3-11

Overexpression analysis of GATA-1 in NIH 3T3 cells

NIH 3T3 cells were transfected with over expression vector encoding mGSTT1 (pLenti6/GATA-1-V5), and incubated for 24h, and used for RT-PCR analyses.

Semi-quantitative RT-PCR analysis of stress responsive genes in those NIH 3T3 cells. The total RNA were isolated from these cells, and RT-PCR analysis was performed using mouse specific primers for GSTs, Bcl2 and Bax. Theses graphs represent the averaged band intensity of those genes with SEM, normalized with β-tubulin from several independent experiments. Statistical analysis was conducted using Student's *t*-test (**P <0.01, *P < 0.05.

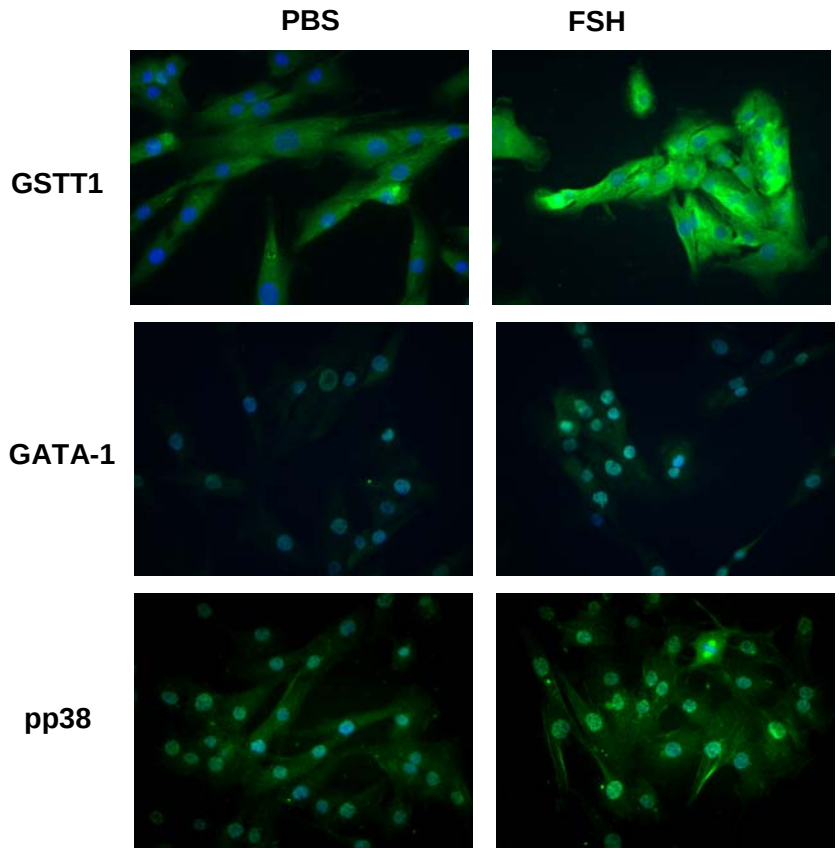


Fig. 3-12

The expression analysis of GSTs in FSH treated KGN cells

The mean fluorescence intensity of GSTT1, GATA-1 and pp38 were measured in KGN cells treated with FSH (100 ng/ml) or PBS for control group and normalized with the number of cells. The cells were counterstained with Hoechst 33342 at 10 μM to visualize the nuclei (blue).

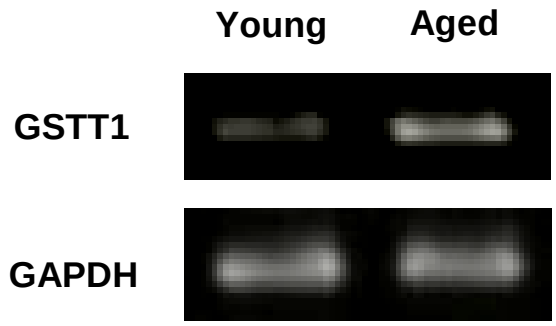


Fig. 3-S1

The expression of GSTT1 in oocytes and granulosa cells (cumulus-oocyte complexes) from young and aged mice

These panels present typical mRNA expression of GSTT1 in oocytes corrected from young (8 weeks-old) and aged (50 weeks old) mice by RT-PCR analysis.

Fig. 3-S1

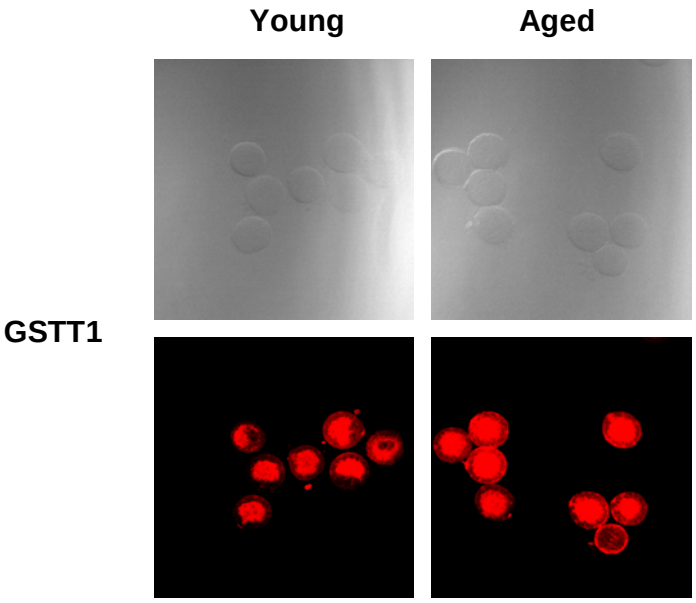


Fig. 3-S2

The expression of GSTT1 in oocytes and granulosa cells (cumulus-oocyte complexes) from young and aged mice

Immunostaining of GSTT1 (red) in oocytes from young 8 weeks of and aged 50 weeks of mice (right panels). Upper panels show the morphology of these oocytes.

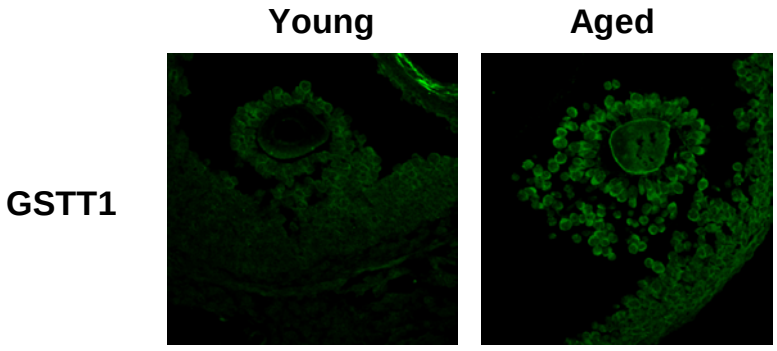


Fig. 3-S3

The expression of GSTT1 in oocytes and granulosa cells (cumulus-oocyte complexes) from young and aged mice

Immunostaining of GSTT1 (green) in cumulus-oocyte complexes (COCs) from young 8 weeks of and aged 50 weeks of mice.

General conclusion

It is now widely accepted that oxidative stress accelerates aging[121]. Age-dependent damage of tissues and cells is exemplified by increases in lipid peroxidation and protein oxidation[101]. As with other cell types, oocytes and ovarian somatic cells are thought to be damaged by ROS, leading to age-related declines in fertility[37]. However, direct evidence that ROS are involved in age-related reproductive failure in humans or other mammals has not yet been provided. The fact that SOD1, SOD2 and catalase, which are ROS scavengers, were down-regulated by aging in human granulosa cells provided some indirect evidence[39]. Also, some genes involved in mitochondrial function and oxidative stress including SOD1 and the thioredoxin family, were found to be expressed differently by aging in mouse oocytes[38]. These findings indicate that excess oxidative stress promotes cellular senescence. In fact, an abundance of ROS is believed to promote apoptosis in ovarian cells, and apoptosis of granulosa cells was reported to be increased by aging[122]. Also, increased mutations in mitochondrial DNA were found in aged oocytes and granulosa cells and were closely related to oocyte quality[123], suggesting that mitochondria-related apoptosis genes could be involved in reproductive aging. In this regard, it is reasonable that gene targeting of Bax, a proapoptotic gene, resulted in delayed ovarian aging[124].

GSTs play a critical role in antioxidants system and protect from ROS by inactivating them and repairing cell damage[48]. In general, aging causes cellular dysfunction and apoptosis via loss of antioxidants; thus, GSTs are expected to be down-regulated by aging[49,50]. However, we demonstrated that GSTT1 expression was up-regulated in aged granulosa cells and renal cells. Reportedly, glutathione peroxidase and GSTT1 were up-regulated in human epidermal cells with aging, suggesting that GSTT1 up-regulation could be due to the concentration of ROS increased with aging and be one of antioxidant defense system in dermis cells [116]. Likewise, up-regulation of GSTT1 in both granulosa cells and renal cells could respond to age rerated-stress and play a role as detoxification enzyme. Therefore, GSTT1 could be sensitive to the age-related stress unlike other GSTs.

Most phase II detoxification enzyme is regulated by Nrf2, a transcription factor. Nrf2 is known to be activated by oxidative-stress, electrophiles, many chemicals and cancer chemopreventive agents, and regulates the expression of various detoxifing and antioxidant genes including GSTs[33,34]. Targeted disruption of Nrf2 in mice (Nrf2KO) have been reported to show decreased expression of most GSTs and the catalytic subunit of glutamate cysteine ligase (GCLC) as compared to wild type mice[31,32,40,41]. Shih et al. reported that age-related declines of antioxidant defense

are closely related the expression level of Nrf2 in rats[42]. MEFs prepared from Nrf2KO showed decreased expression of GSTs, although GSTT1 expression was not changed compared to wild type mice. In addition, GSTT1 expression was not suppressed by Nrf2 inhibitor, all-trans retinoic acid (ATRA), which interferes with binding of Nrf2 to the ARE. Conversely, ATRA seemed to induce GSTT1 expression in KGN cells. In fact, ATRA-induced apoptosis in gastrointestinal stromal tumor (GIST-T1) cells was accompanied by the down-regulated expression of survivin and up-regulated expression of Bax protein [75]. Moreover, compared to other phase II detoxification enzymes, several mutations were identified in the ARE tandem core of GST theta class. Thus, the induction of GSTT1 could not be associated with Nrf2, suggesting that there is other signaling pathways involved in the regulation of GSTT1.

A variety of upstream signaling pathways have been reportedly implicated in the induction of phase II detoxification enzyme genes. These include extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), and phosphatidylinositol 3-kinase (PI3K). Even for the induction of a single gene, various upstream signaling pathways are involved[55]. It has been suggested that the upstream signaling pathways involved in the induction of Nrf2 regulating genes may be affected by gene, inducer and cell type.

Among the these kinase, ERK is mainly activated by mitogens and growth factors, while p38 MAPK and JNK are activated by many environmental stress stimuli including oxidative stress defense response[56,57]. Ito et al. reported that p38 was activated by FSH in KGN cells[67], and my results demonstrated that GSTT1 expression was also induced by FSH in KGN cells. Furthermore, a p38 MAPK inhibitor was shown to promote the production of steroidogenic acute regulatory protein by FSH and to reduce the expression of P450arom in rat granulosa cells[70]. Because GSTT1 expression was suppressed by p38 MAPK inhibitor, GSTT1 and p38 MAPK would affect this kind of signaling.

Various transcription factors have been reported to be involved in the activation of p38 MAPK signaling, and Nrf2 is exactly one of such factors[55,80]. Others include ATF-1/2, Sap1,GADD153, GATA factors, and p53[89-96]. Among them, GATA family of six zinc finger transcription factors specifically binds to a consensus DNA sequence (A/T)GATA(A/G) in gene promoters and enhancers[97]. In granulosa cells, GATA-4 is induced by FSH and involved in protein kinase A and p38 MAPK pathway activated by gonadotropins and cAMP (Fig.3-1)[102,103]. GATA-1 also exhibit gonadal expression especially in Sertoli cells and Leydig cells[105], suggesting the association with FSH. The rat FSH receptor promoter possesses a functional

GATA-1 site[106]. In the present study, I demonstrated that overexpression and siRNA-mediated knockdown of GSTT1 dramatically swung the expression of GATA factors; I also showed that induction of GATA-1 up-regulated GSTT1 expression in NIH 3T3 cells. GATA-1 is expressed in hematopoietic cells and required for the development of megakaryocytes and erythroid cells. Therefore, it is believed to be a key regulator of erythropoiesis[98]. There is the evidence that megakaryocytes obtained from 13.5 days GATA-1 deficient C57BL/6 embryos scarcely expressed GSTT1, suggesting that regulation of GSTT1 could involve GATA-1. However, GATA-1 expression is barely expressed in ovarian cells differently from testicular cells; it is not clear whether GSTT1 is induced by GATA-1 in granulosa cells with aging. Tissue-specific overexpression and siRNA-mediated knockdown studies are needed to resolve this problem.

In addition, the overexpression studies of GSTT1 and GATA-1 denote that Bax shows the same expression pattern as GSTT1. These results suggest that GSTT1 and Bax could have the similar stress-response and signaling cascade, even though their function might be different.

In this study, I demonstrated that GSTT1 was not induced by Nrf2-ARE-mediated pathway, and more likely to be associated with GATA factors, downstream of p38 MAPK in some somatic cells. GSTT1 may have different

stress-response and stress-signaling cascades from other GSTs.

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