Inhibition of GATA2-dependent transactivation of the *TSHβ* gene by ligand-bound estrogen receptor α

Koji Nagayama, Shigekazu Sasaki, Akio Matsushita, Kenji Ohba, Hiroyuki Iwaki, Hideyuki Matsunaga, Shingo Suzuki, Hiroko Misawa, Keiko Ishizuka, Yutaka Oki, Jaeduk Yoshimura Noh[1] and **Hirotoshi Nakamura**

Second Division, Department of Internal Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu, Shizuoka 431-3192, Japan
[1]Ito Hospital, 4-3-6 Jingumae, Shibuya-ku, Tokyo, 150-8308, Japan
(Correspondence should be addressed to S Sasaki; Email: sasakis@hama-med.ac.jp)

Abstract

Transcriptional repression of the TSH-specific β subunit (*TSHβ*) gene has been regarded to be specific to thyroid hormone (tri-iodothyronine, T_3) and its receptors (TRs) in physiological conditions. However, *TSHβ* mRNA levels in the pituitary were reported to decrease in the administration of pharmacologic doses of estrogen (17-β-estradiol, E_2) and increase in E_2 receptor (ER)-α null mice. Here, we investigated the molecular mechanism of inhibition of the *TSHβ* gene expression by E_2-bound E_2-estrogen receptor 1 (E_2-ERα). In kidney-derived CV1 cells, transcriptional activity of the TSHβ promoter was stimulated by GATA2 and suppressed by THRBs and ERα in a ligand-dependent fashion. Overexpression of PIT1 diminished the E_2-ERα-induced inhibition, suggesting that PIT1 may protect GATA2 from E_2-ERα targeting by forming a stable complex with GATA2. Interacting surfaces between ERα and GATA2 were mapped to the DNA-binding domain (DBD) of ERα and the Zn finger domain of GATA2. E_2-dependent inhibition requires the ERα amino-terminal domain but not the tertiary structure of the second Zn finger motif in E_2-ERα-DBD. In the thyrotroph cell line, TαT1, E_2 treatment reduced *TSHβ* mRNA levels measured by the reverse transcription PCR. In the human study, despite similar free thyroxine levels, the serum TSH level was small but significantly higher in post- than premenopausal women who possessed no anti-thyroid antibodies (1·90 μU/ml ± 0·13 s.e.m. vs 1·47 μU/ml ± 0·12 s.e.m., $P<0·05$). Our findings indicate redundancy between T_3-TR and E_2-ERα signaling exists in negative regulation of the *TSHβ* gene.
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Introduction

Thyrotropin (thyroid-stimulating hormone, TSH) is a heterodimer consisting of the TSH-specific β subunit (TSHβ) and the chorionic gonadotropin α chain (CGA) that is common to luteinizing hormone (LH), follicle-stimulating hormone (FSH), and chorionic gonadotropin. Thyroid hormone (tri-iodothyronine, T_3)-mediated negative feedback of TSH production in the pituitary is a central mechanism of the pituitary–thyroid axis and is believed to be specific to T_3 at physiological concentrations. However, administration of pharmacologic doses of estrogen (17-β-estradiol, E_2) was known to augment the effect of thyroid hormone replacement to suppress *TSHβ* and *CGA* mRNA (Wondisford 1996 and references therein). Indeed, E_2 inhibits the upregulation of *Tshβ* and *Cga* mRNA in the pituitary of hypothyroid rats (Franklyn *et al.* 1987). Bottner & Wuttke (2005) and Bottner *et al.* (2006) reported that ovariectomy increased pituitary *TSHβ* mRNA levels and that this elevation was abolished by 17β-E_2-3-benzoate treatment. Boado *et al.* (1983) showed that E_2 benzoate induced a marked depression of intrapituitary TSH. Sekulic *et al.* (1998) demonstrated that E_2 treatment reduced the number of TSHβ-positive cells in the rat pituitary.

There are two types of estrogen receptors (ERs), ERα and ERβ. In the anterior lobe of the pituitary, ERα is the major receptor (Kuiper *et al.* 1996, Pelletier *et al.* 2000, Liu & Cui 2005) and is expressed in thyrotrophs as well as gonadotrophs (Stefaneanu *et al.* 1994, Gittoes *et al.* 1997). Interestingly, Scully *et al.* (1997) reported that, in the pituitary of ERα-deficient mice, mRNA levels for not only *Lhβ* and *Fshβ* but also *Tshβ* and *Cga* were dramatically elevated. Compared with wild-type mice, the amount of mRNA for *Tshβ* and *Cga* in *Esrα* null mice increased by 3·20- and 4·36-fold respectively. Immunostaining using specific antibodies revealed that the number of TSHβ- and CGA-positive cells also increased (Scully *et al.* 1997). These findings are similar to observations in mice devoid of all known T_3 receptors (TRs; Gothe *et al.* 1999). Although these observations suggest that E_2 suppresses TSHβ expression via ERα, the molecular mechanism has not been clarified.

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ER and TR belong to the nuclear hormone receptor superfamily and share a basic structure consisting of a receptor-specific amino-terminal domain (NTD), central DNA-binding domain (DBD), and carboxyl (C)-terminal ligand-binding domain (LBD; Mangelsdorf *et al.* 1995). In the promoter region of target genes whose transcription is enhanced by treatment with E_2 or T_3, ER homodimers and TR heterodimers formed with the retinoid X receptor (RXR) recognize the E_2-response element (ERE) and the T_3-response element (TRE) respectively. The tertiary structure of DBDs is maintained by two Zn finger motifs, and the amino acid sequences of the P- and D-boxes of DBD play critical roles in the differential recognition of ERE and TRE (Umesono & Evans 1989). E_2-bound ER (E_2-ER) and T_3-bound TR (T_3-TR) recruit coactivators such as members of the p160 family or CREB-binding protein (CREB)/p300, whose histone acetyl-transferase activities relax the chromatin structure and result in the enhancement of transcription (Perissi & Rosenfeld 2005). In addition, T_3-TR (Ito & Roeder 2001) and E_2-ER (Zhang *et al.* 2005) recruit TRAP220, a component of the TRAP/SRB/MED-containing cofactor complex of the RNA polymerase II holo-enzyme. In the absence of T_3, TR recruits co-repressors such as nuclear receptor co-repressor or the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT). These co-repressors associate with histone deacetylases (HDACs), resulting in repression of transcription (Perissi & Rosenfeld 2005).

Of note, TR and ER have the potential to bind an identical half-site sequence, AGGTCA, although the number of spacing nucleotides between the half-sites and their orientations are different between TRE and ERE (Mangelsdorf *et al.* 1995); ERE can be recognized by the TR monomer, in addition to homodimer and heterodimer with RXR (Klinge *et al.* 1997). T_3-TR can also directly bind to ERE of the promoter for the progesterone receptor gene and stimulate its transcription (Scott *et al.* 1997). On the other hand, when TR and ER coexist, T_3-TR was reported to inhibit E_2-ERα-mediated transactivation of the promoters for the preproenkephalin gene (Zhu *et al.* 1996, Vasudevan *et al.* 2001*b*) and the prolactin gene (Pernasetti *et al.* 1997), probably via squelching of coactivators common to both receptors. Similar mutual inhibition has been reported between T_3-TR and other ligand-bound steroid hormone receptors (Zhang *et al.* 1996). Thus, the redundancy of DNA recognition and the common utilization of cofactors have been postulated to mediate crosstalk between the E_2-ER and T_3-TR signaling pathways (Vasudevan *et al.* 2001*a*, 2002).

In the mouse TSHβ promoter, the DNA sequence between nt −271/−80 (corresponding to the sequence between nt −269/−78 in the human *TSHβ* gene) was reported to be sufficient for maximal promoter activity in thyrotrophs (Wood *et al.* 1990). In this promoter region, there are binding sites for two transcription factors, PIT1 and GATA2 (Haugen *et al.* 1996, Gordon *et al.* 1997, 2002, Dasen *et al.* 1999, Charles *et al.* 2006). Co-existence of PIT1 and GATA2 in thyrotrophs is essential for the expression of the *TSHβ* gene (Gordon *et al.* 1997, Dasen *et al.* 1999). PIT1 is a pituitary-specific transcription factor expressed in somatotrophs, lactotrophs, and thyrotrophs. GATA2 is a subtype of the GATA family of transcription factors and binds with the GATA-responsive element (GATA-RE) through its Zn finger domain, which has high homology among all GATA family members (Ferreira *et al.* 2005). It was recently reported that TRAP220, which is a coactivator for liganded TR and ER, also functions as a coactivator for GATA2 and PIT1 (Gordon *et al.* 2006) and plays an important role in the transactivation of the *TSHβ* gene (Ito *et al.* 2000). In addition, TRAP220 was reported to be a coactivator for GATA1 (Stumpf *et al.* 2006), transactivation by which is known to be inhibited by E_2-ERα (Blobel *et al.* 1995, Blobel & Orkin 1996).

Using non-pituitary CV1 cells, we recently reported that T_3-TRβ2 represses the expression of the *TSHβ* gene driven by GATA2 and PIT1 through interference of GATA2-induced transactivation, and that, contrary to the previous reports, the negative TRE (nTRE; Wondisford *et al.* 1989) and negative regulatory elements (NRE; Sasaki *et al.* 1999) were not required (Matsushita *et al.* 2007). The reporter assay using CV1 cells provides an ideal experimental platform to investigate the difference between positive and negative regulation by T_3, since this cell line is one of those most frequently used for the study of positive regulation (Nakano *et al.* 2004). We found that THRB-DBD directly interacts with the Zn finger domain of GATA2 (GATA2-Zf) and that T_3-TR targets TRAP220, which functions as a coactivator for GATA2 in the context of the TSHβ promoter (Matsushita *et al.* 2007). Reflecting T_3-specificity *in vivo*, ligand-bound retinoic acid receptor, vitamin D receptor, RXR, or peroxisome proliferator-activated receptor γ2 all did not exhibit negative regulation of the TSHβ promoter in our experimental system (Nakano *et al.* 2004). Unexpectedly, however, E_2-ERα exhibited significant suppression of the TSHβ promoter although the magnitude of suppression (39·4%±4·5 S.E.M.) was less than that of T_3-TRβ2 (64·1%±2·6 S.E.M.; Nakano *et al.* 2004). Here, we report that, in the negative regulation of the *TSHβ* gene, there is a redundancy between E_2-ERα- and T_3-TR-mediated signaling pathways.

Materials and Methods

Plasmid construction

We recently found that not only T_3-TR (Tillman *et al.* 1993, Maia *et al.* 1996) but also E_2-ERα have a tendency to suppress firefly luciferase-based reporter gene (data not shown). Thus, we employed the CAT-based reporter system. The *TSHβ-CAT* reporter gene was constructed by fusing the human TSHβ promoter (nt. −128/+37) with the *CAT* reporter gene, whose backbone lacks the pUC-derived AP-1 site (Sasaki *et al.* 1999, Nakano *et al.* 2004). TSHβ-D4-CAT was reported previously (Matsushita *et al.* 2007). The DNA fragment encompassing the promoter region for *TSHβ* gene

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(nt. 615/129) was amplified using PCR from human genomic DNA as template and subcloned into EcoRI site in *TSHβ-CAT* to generate *TSHβ (615/+37)-CAT*. In *TSHβ-M1-CAT*, the putative nTRE/NRE remained intact (data not shown). The expression plasmids for mouse *Gata2* (pcDNA3-mGATA2), human *PIT1* ($pCB6^+$-hPit1), wild-type human *TRβ1* (pCMX-hTRβ1), *TRβ1*-deletion mutants (pCI-C1 and C2), wild-type rat *Trβ2* (pCMX-rTRβ2), and human *ERα* were described previously (Nakano *et al.* 2004). The deletion mutants of human *ERα* (Δ170 and Δ247) and mutant *ERα* such as C205S, C221G, and C240S were generated from wild-type *ERα* using standard subcloning techniques or a site-directed mutagenesis kit (Stratagene). The plasmid expressing the recombinant protein of the GATA2 Zn finger domain (GATA2-Zf) combined with glutathione-*S*-transferase (GST), pGEX-4T-1-GATA2-Zf, was described elsewhere (Matsushita *et al.* 2007). All constructs were confirmed by DNA sequencing.

Cell culture and transient transfection

CV1 cells were grown in monolayers and cultured at 37 °C under CO_2/air (1:19) in Dulbecco's modified Eagle's medium (DMEM) containing 10% dextran–charcoal-stripped fetal bovine serum (10% DCC serum), penicillin G (100 units/ml), and streptomycin (100 μg/ml). TαT1 cells, a mouse thyrotroph cell line (Yusta *et al.* 1998), were seeded on Matrigel-coated plates (Becton Dickinson Labware, Bedford, MA, USA). The cells were maintained under the same conditions as CV1 cells. The CV1 cells were trypsinized and plated in 60 mm diameter dishes for 24 h before transient transfection using the calcium phosphate technique (Sasaki *et al.* 1995). The cells at a density of 10^6 cells/plate were transfected with 0·6 μg of the expression plasmids for *Esrα* or *Thrb* along with 4·0 μg of the *Tshβ-Cat* reporter gene, 1·8 μg β-galactosidase expression vector pCH111 (a modified version of pCH110; Pharmacia LKB Biotechnology), 0·1 μg human *PIT1* expression vector ($pCB6^+$-hPit1), and 0·4 μg mouse *Gata2* expression vector (pCDNA3-mGATA2), and the pCMX empty vector as carrier DNA (7·2 μg DNA per dish in total). After the cells were exposed to the calcium phosphate/DNA precipitates for 20 h, the medium was replaced with phenol red-free DMEM containing 5% (v/v) and the cells were allowed to grow for an additional 24 h with or without 1 μM E_2 or T_3. After incubation for an additional 24 h, the cells were harvested and CAT activity was measured as described previously (Sasaki *et al.* 1995). The transfection efficiency was normalized by β-galactosidase assay. For each CAT reporter assay, we performed transfection with CAT reporter gene driven by cytomegalovirus promoter (CMV-CAT; 40 ng/dish), the magnitude of which was adjusted to the value of 100. Immunoblotting with anti-ERα antibody (1:200, cat. sc-8002, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was performed using a standard method described previously (Matsushita *et al.* 2007).

GST pull-down assay

Escherichia coli (DH5α) transformed with pGEX-4T-1-GATA2-Zf were induced with 0·1 mM isopropyl-1-thio-β-galactopyranoside for 4 h. The *E. coli* pellet was sonicated and the fusion proteins were mixed with glutathione-sepharose beads (Amersham Pharmacia Biotech) for purification. Receptor proteins were translated *in vitro* using rabbit reticulocyte lysate (Promega Corp.) in the presence of ^{35}S-methionine. Radio-labeled receptors were incubated with GST protein fused to GATA2-Zf (GST-Zf) in the binding buffer (150 mM NaCl, 20 mM Tris–HCl (pH 7·5), 0·3% Nonidet P-40, 1 mM dithiothreitol, 0·5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin and leupeptin) for 3 h at 4 °C. The precipitants were washed three times with the binding buffer. Bound protein was analyzed by 10% SDS-PAGE and visualized using the FLA-3000 autoradiography system (Fujifilm, Tokyo, Japan).

Gel shift assay

Oligonucleotides for vit-A2 consensus ERE (Scott *et al.* 1997; sense, 5′-AATTCGTCCAAAGTCAGGTCACAGTGACCTGATCAAAGTT-3′; antisense, 5′-AACTTTGATCAGGTCACTGTGACCTGACTTTGGACGAATT-3′) were labeled with γ^{32}P-ATP using thyroxine (T_4)-polynucleotide kinase (Toyobo, Tokyo, Japan). The receptor proteins were translated *in vitro* using a TNT T7 quick-coupled transcription/translation system (Promega Corp). The radio-labeled probes and *in vitro*-translated receptors were incubated for 20 min at room temperature in 20 μl binding buffer containing 10 mM HEPES–NaOH (pH 7·9), 50 mM KCl, 0·1 mM EDTA, 10% glycerol, 0·5 mM dithiothreitol, and 0·1 mg/ml poly (dI-dC). The DNA–protein complexes were resolved on 5% polyacrylamide gels at 150 V for 1·5 h. Gels were dried and visualized using the FLA-3000 autoradiography system.

RNA isolation and real-time reverse transcription-PCR (RT-PCR)

TαT1 cells cultured in 10% DCC serum were incubated in the presence of 1 μM E_2 or T_3 for 48 h. The cells were harvested and total RNAs were purified by the acid guanidium thiocyanate–phenol–chloroform extraction method (Kawai *et al.* 2004). For the first-strand cDNA synthesis, 2 μg total RNA were mixed with random hexanucleotides and 200 units Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp). The cDNA for *TSHβ* was amplified with the forward primer (5′-GGCAAACTGTTTCTTCCCAA-3′) and the reverse primer (5′-TCTGTGGCTTGGTGCAGTAG-3′). The cDNA for glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified with the forward primer (5′-TGAACGGGAAGCTCACTGG-3′) and the reverse primer (5′-TCCACCACCCTGTTGGCTGTA-3′). PCR amplification was carried out using a DNA thermal cycler (Takara Bio Inc., Shiga, Japan) under the following

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conditions: denaturation at 95 °C for 1 min, annealing at 62 °C for 1 min, extension at 72 °C for 2 min, and the final extension at 72 °C for 4 min. We determined the cycle number for each primer set so that the specific product was amplified during the exponential phase of the amplification. Based on preliminary studies (data not shown), 27 cycles were employed for the amplification of *TSHβ* and *GAPDH* cDNA. The PCR products were subjected to electrophoresis on a 1·4% agarose gel and stained with ethidium bromide. Using the SYBR Green I kit and Light Cycler (Roche Diagnostics), precipitated DNA was quantified by real-time PCR using the primers mentioned above. The thermal cycling conditions were 10 min at 95 °C, followed by 27 cycles of 10 s at 95 °C for denaturing, 10 s at 62 °C for annealing, and 7 s at 72 °C for extension. PCR signals were analyzed using Light Cycler software Ver. 3.5 (Roche Diagnostics).

Measurement of serum TSH, free T_4, and E_2 in pre- and postmenopausal women

Among female patients who visited Ito Hospital in May 2004, 134 subjects with simple goiter or thyroid nodule(s) and no thyroid autoantibodies against thyroglobulin, thyroid peroxidase, or TSH receptor were extracted. They were divided into 62 premenopausal women and 72 postmenopausal women. The premenopausal group consisted of women between 30 and 50 years old (37·35±5·40 years old) and their serum E_2 levels were all higher than 20 pg/ml. The postmenopausal group included women between 50 and 70 (59·29±4·70) years old with serum E_2 levels lower than 20 pg/ml. TSH and free T_4 were measured with Roche ECLusys kit (Roche) and E_2 with electro chemiluminescence immunoassay (SRL, Tachikawa, Japan).

Statistical analysis

Each experiment was performed in duplicate more than three different times and each result is expressed as the mean±S.E.M. Using StatView 4.0 software (Abacus Concepts Inc., Berkeley, CA, USA), we examined statistical significance in Figs 6 and 7 by Mann–Whitney *U* test. All other statistical analyses were performed using one-way ANOVA followed by Fisher's protected least significant difference test. $P<0·05$ was considered significant.

Results

TSHβ promoter activity is inhibited by not only T_3-TRβ2 but also E_2-ERα

To explore the molecular mechanism underlying the E_2-dependent inhibition of the *TSHβ* gene expression, we carried out a reporter assay using *TSHβ-CAT* (Fig. 1A), in which the human *TSHβ* promoter (nt. 128/+37) is fused to the *CAT* reporter gene. In accordance with previous reports (Gordon *et al.* 1997, Dasen *et al.* 1999), co-expression of PIT1 and GATA2 transactivated the *TSHβ* promoter in CV1 cells (Fig. 1B, lane 2). This activity was significantly repressed by not only T_3-TRβ2 but also E_2-ERα (lanes 2, 4, and 6). When cognate receptors were co-expressed with PIT1 and GATA2, T_3 and E_2 inhibited transcription by 64·1%±2·6 S.E.M. (lanes 3 and 4) and 39·4%±4·5 S.E.M. (lanes 5 and 6) respectively. E_2-ERα-induced repression depended on the E_2 concentration (Fig. 1C) and the expression level of ERα (Fig. 1D). The inhibition of the *TSHβ* promoter by E_2-ERα was observed in Hela and 293T cells (data not shown). We also examined the effect of E_2-ERα on the *TSHβ* (−615/+37)-*CAT* reporter gene, which has longer *TSHβ* promoter encompassing nt. −615/+37, and found again that the transcriptional activity in CV1 cells was stimulated by PIT1 and GATA2, and significantly decreased by 1 μM E_2 (Fig. 1E).

Overexpression of PIT1 relieves E_2-dependent inhibition of the TSHβ gene and E_2-ERα targets GATA2-induced transactivation

We tested the effect of PIT1 overexpression on the negative regulation of the *TSHβ* promoter by E_2-ERα. As shown in Fig. 2A, inhibition by E_2-ERα was abolished by increased levels of PIT1, suggesting that a large amount of PIT1 antagonizes the E_2-ERα-dependent inhibition of the *TSHβ* gene. We recently found that deletion of a short sequence between GATA-REs and the TATA-box in the *TSHβ* promoter (nt. 82/52; Fig. 1A) enabled GATA2 alone to transactivate without PIT1 (Matsushita *et al.* 2007), and we designated this deleted sequence as the suppressor region (SR). The construct TSHβ-M1-CAT (Fig. 2B, left panel) in which SR was deleted was activated by GATA2 alone, and this activity was inhibited by E_2-ERα (Fig. 2B, right panel). GATA2 alone without PIT1 transactivated two other reporter constructs, *TSHβ-D4-CAT*, in which nTRE/NRE in *TSHβ-M1-CAT* was mutated (Matsushita *et al.* 2007), and CGA-CAT, which possesses a functional GATA-RE (Steger *et al.* 1994). Again, E_2-ERα repressed the GATA2-induced transactivation of TSHβ-D4-CAT and CGA-CAT by 53·1%±1·9 S.E.M. and 30·5%±6·2 S.E.M. respectively (data not shown). Collectively, transactivation by GATA2 alone was sufficient to mediate negative regulation by E_2-ERα and PIT1 antagonized this inhibition.

The Zn finger domain of GATA2 directly interacts with TR and ERα in vitro

The amino acid sequence of the Zn finger domain of GATA2 (GATA2-Zf), but not the N- or C-terminal region, has high homology with that of GATA1 (Fig. 3A), and both GATAs are known to transactivate the same GATA-RE. In addition, E_2-ERα was reported to inhibit GATA1-induced transactivation (Blobel *et al.* 1995). As predicted, GATA1-induced transactivation of *TSHβ-CAT* was also repressed by

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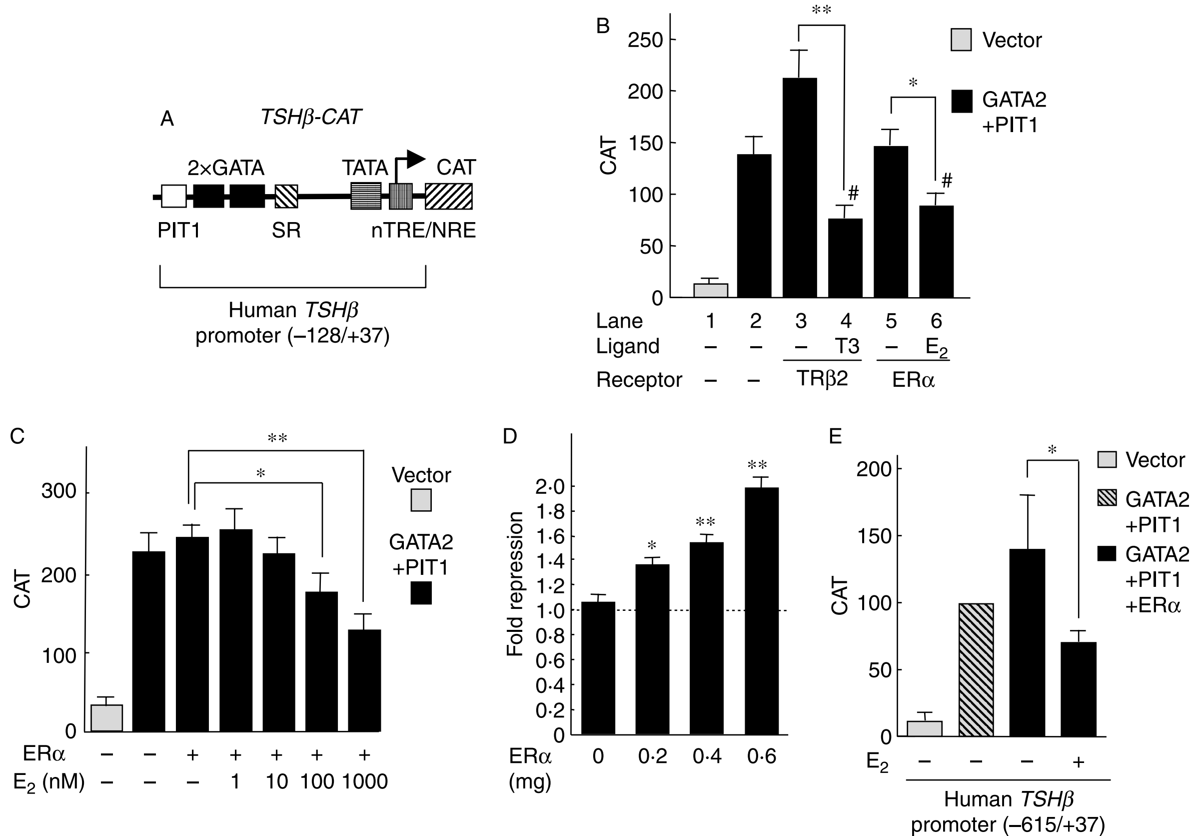

Figure 1 Effect of E_2-ERα on the *TSHβ* promoter activity stimulated by PIT1 and GATA2. (A) Schematic of the *TSHβ-CAT*. The binding sites for PIT1 and GATA2, suppressor region (SR), TATA box, and reported nTRE/NRE are indicated as boxes. (B) Using the calcium phosphate method, the expression plasmids for ERα or TRβ2 (0·6 μg) were co-transfected with *TSHβ-CAT* (4·0 μg), PIT1 (0·1 μg), and GATA2 (0·4 μg) into CV1 cells in the absence or presence of 1 μM E_2 or T_3 respectively. After incubation for 24 h, the cells were harvested and the CAT activity was measured. The CAT activity of CMV-CAT is represented as 100%. The data are shown as the mean ± s.e.m. of at least six individual experiments. *$P<0·05$; **$P<0·01$; $^{\#}P<0·05$ versus PIT1 and GATA2. (C) *TSHβ-CAT* was co-transfected with PIT1, GATA2, and ERα into CV1 cells in the presence of 0–1 μM E_2. The CAT activity of CMV-CAT is represented as 100% *$P<0·05$; **$P<0·01$. (D) Increasing amounts of the ERα expression plasmid (0–0·6 μg/6 cm dish) were co-transfected with *TSHβ-CAT* (4·0 μg) and the expression plasmid for PIT1 (0·1 μg) and GATA2 (0·4 μg) into CV1 cells. The magnitude of CAT activity without E_2 was divided by that with E_2 (1 μM) to calculate the fold repression. The results are shown as the means ± s.e.m. from six independent experiments. *$P<0·05$; **$P<0·01$. (E) The expression plasmids for ERα (0·6 μg) were co-transfected with *TSHβ (−615/+37)-CAT* (4·0 μg), PIT1 (0·1 μg), and GATA2 (0·4 μg) into CV1 cells in the absence or presence of 1 μM E_2. All results in (E) are shown as means ± s.e.m. for eight independent experiments. *$P<0·05$. The CAT activity of CMV-CAT is represented as 100%.

E_2-ERα (Fig. 3B). While C-terminal finger of GATA1 directly recognizes the GATA-RE, the N-terminal finger is known to interact with Friend of GATA (FOG) 1 and 2 that are the strong co-repressor for the GATA family transcription factors. We reported (Matsushita *et al*. 2007) that transactivation by a mutant GATA2, GATA2-C295A (Fig. 3A), which has an amino acid substitution from cysteine to alanine at codon 295 of the N-terminal Zn finger, was resistant to the inhibition by T_3-TRβ2. The finding that the basal transcription by C295A was significantly higher than that by empty vector (Fig. 3C) indicates that the overall structure as functional GATA2 is partly preserved in this mutant. However, transactivation induced by GATA2-C295A was not inhibited by E_2-ERα. These findings indicate that interaction between GATA2-Zf and ERα is essential to E_2-ERα-mediated inhibition. Using the GST fusion protein with GATA2-Zf (GST-Zf, Fig. 3A), we tested the direct interaction between ERα and GATA2-Zf *in vitro*. As reported previously (Matsushita *et al*. 2007), GST-Zf interacted with TRβ1 (Fig. 3D, upper panel) but not the luciferase protein (lower panel). In an E_2-independent manner, GST-Zf also bound to radio-labeled full-length ERα (Fig. 3D, middle panel). As shown in Fig. 3E, the mutant ERαs N2 and Δ247, which lacked DBD, did not interact with GST-Zf, suggesting that ERα-DBD binds to GATA2-Zf as in the case of THRB-DBD.

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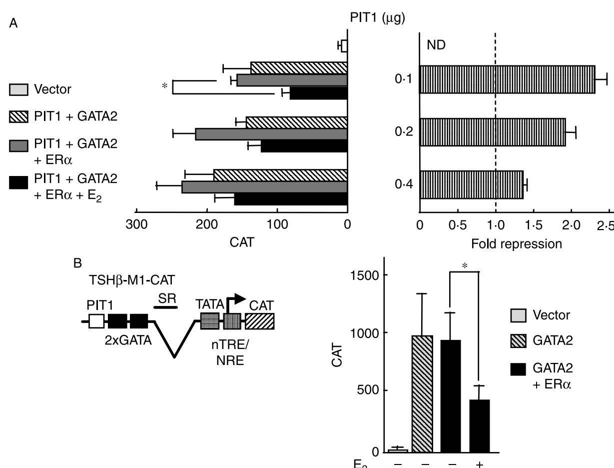

Figure 2 Roles of PIT1 in the negative regulation of *TSHβ* gene expression by E_2-ERα (A) TSHβ-CAT (4·0 μg) was transfected into CV1 cells together with the expression plasmids for PIT1 (0·1–0·4 μg), GATA2 (0·4 μg), and ERα (0·6 μg) in the absence or presence of 1 μM E_2 (left panel). The fold repression (right panel) was calculated from CAT activity without E_2 divided by that with 1 μM E_2. (B) Schematic of TSHβ-M1-CAT (left panel). The suppressor region (SR) was deleted in this construct. TSHβ-M1-CAT was co-transfected with the expression plasmids for ERα, GATA2 into CV1 cells in the absence or presence of 1 μM E_2. The results are shown as means±S.E.M. from six experiments. $*P<0·05$.

The E_2-ERα-induced inhibition of the TSHβ promoter does not require the second Zn finger motif

P- and D-boxes and the zinc finger structure in DBD are critical constituents that determine receptor specificity in ligand-dependent positive regulation (Mangelsdorf *et al.* 1995). To investigate the involvement of these motifs in the negative regulation of the *TSHβ* gene, we generated three mutant ERαs: C205S in the P-box and C221G and C240S in the stem of the second Zn finger (Fig. 4A, upper panel). Western blot analysis with an anti-ERα antibody indicated that expression levels of these mutants in CV1 cells were comparable (lower panel). As predicted, these mutants lost DNA-binding affinity as demonstrated by gel shift assay with vitellogenin A2-derived ERE (vit-A2-ERE; Scott *et al.* 1997; Fig. 4B), and failed to activate the *ERE-tk-CAT* reporter gene, in which vit-A2 ERE is fused to the *CAT* reporter gene driven by the thymidine kinase (tk) promoter (Fig. 4C, left panel). As shown in the right panel of Fig. 4C, negative regulation of the *TSHβ* promoter induced by PIT1 and GATA2 was abolished in C205S and C221G. The findings that C205S and C221G directly interacted with GATA2-Zf (Fig. 4D) suggest that physical interaction of ERα-DBD with GATA2 is not sufficient for inhibition by E_2-ERα. Unexpectedly, another mutant ERα, C240S (Fig. 4A) preserved E_2-induced negative regulation of the *TSHβ* gene (Fig. 4C) as well as *in vitro* binding with GATA2-Zf (Fig. 4D), suggesting that the tertiary structure of the second Zn finger motif, which is necessary for ERE recognition, is dispensable for inhibition of the *TSHβ* gene.

The E_2-induced inhibition of the TSHβ promoter requires ERα-NTD

We previously reported that TRβ1-NTD is dispensable for the negative regulation of the TSHβ promoter (Nakano *et al.* 2004). We wanted to compare the function of NTDs of ERα. In contrast to TRβ1, the ERα-NTD truncation mutant Δ170 (Fig. 5A, middle upper panel) did not inhibit but rather stimulated the transcription (right panel), although it mediated positive regulation (left panel). These data suggest that ERα-NTD is critical for inhibition by E_2. As predicted, ERα (Δ247), which lacks the DBD, did not exhibit ligand-dependent transactivation or inhibition. The expression levels of mutant ERαs were comparable (middle lower panel).

As shown in Fig. 5B, the amino acid sequences between ERα and ERβ were conserved in their DBD (96%) and LBD (58%) but not in NTDs (McInerney *et al.* 1998). We found that repression of the *TSHβ* promoter by E_2-ERβ was impaired and the effect of E_2 was not statistically significant (Fig. 5C). To exclude the possibility that ERα-LBD may communicate with the NTD of the same ERα molecule, we tested the function of the chimeric ERα, βNαC, in which the NTD was substituted with that of ERα (Yi *et al.* 2002; Fig. 5B). Again, βNαC failed to mediate E_2-dependent repression (Fig. 5D). Western blotting with an antibody against the C-terminal region of ERα indicated that expression levels of wild-type ERα and βNαC were comparable (Fig. 5E). Together, these findings indicate that the ERα-NTD has an important role in E_2-dependent inhibition.

Effect of E_2 on the expression of the TSHβ gene in thyrotroph cell line TαT1

Serum E_2 concentration is known to influence not only the level of T_4-binding globulin (TBG; Surks & Sievert 1995) but also the production of T_3 and T_4 from the thyroid gland (Furlanetto *et al.* 1999, Sosic-Jurjevic *et al.* 2005, Alotaibi *et al.* 2006, Lima *et al.* 2006). Using cultured thyrotroph cell line TαT1 (Yusta *et al.* 1998), we studied the direct effect of E_2 on the *TSHβ* mRNA level. When *TSHβ* mRNA was detected by conventional RT-PCR, not only T_3 but also E_2 reduced the intensity of the *TSHβ* mRNA band (Fig. 6A). We further measured *TSHβ* mRNA expression by real-time quantative RT-PCR (Fig. 6B). Although the magnitude was smaller than that for T_3, E_2 treatment also significantly reduced the level of *TSHβ* mRNA (67·2%±12·1 S.E.M. vs 49·5%±14·9 S.E.M., $P<0·05$). The magnitudes of suppression were correlated to those from the reporter assay using *TSHβ-CAT* (Fig. 1B).

Comparison of serum TSH levels between pre- and post-menopausal women

To evaluate the clinical significance of E_2 effects on serum TSH, we compared the serum TSH level of 72 postmenopausal women with that of 62 premenopausal women,

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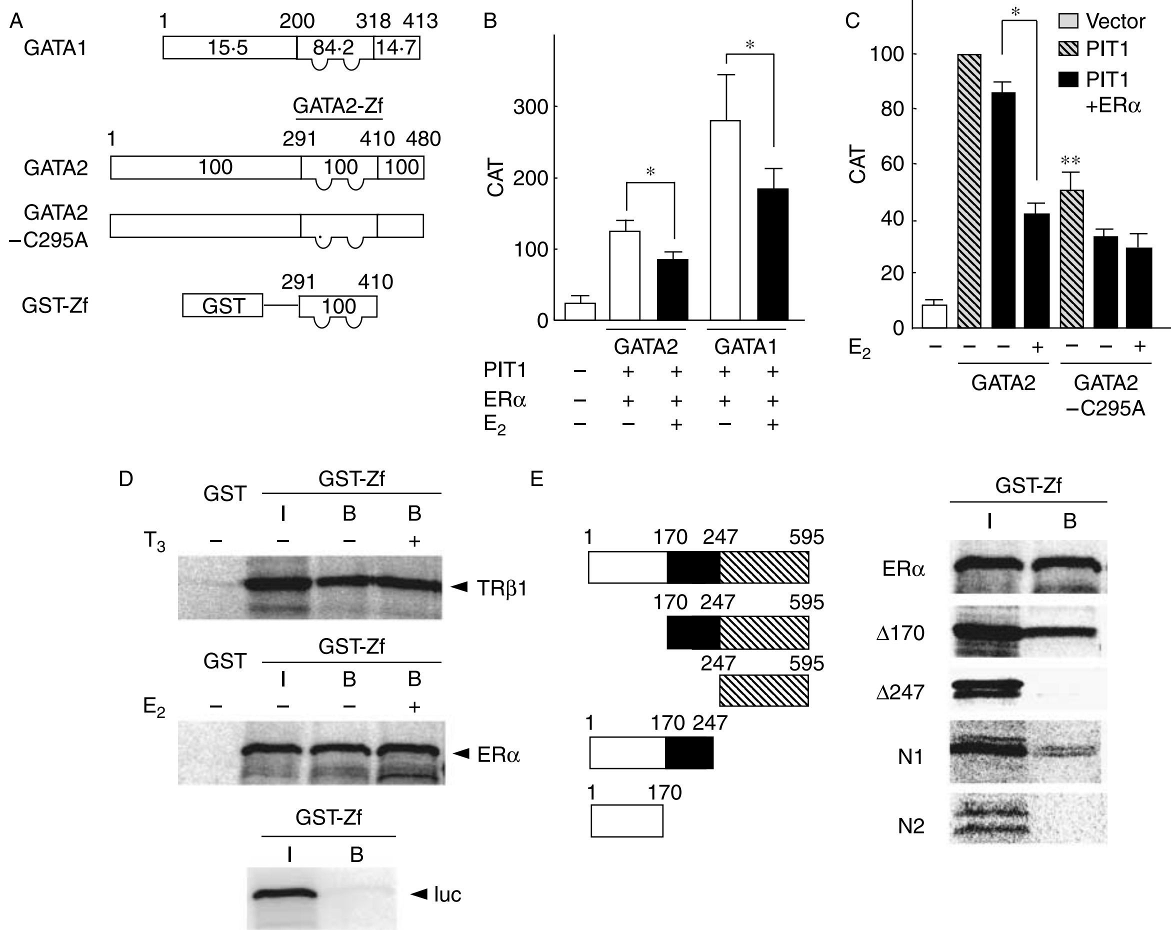

Figure 3 The interaction between GATA2-Zf and ERα-DBD. (A) Schematic of GATA1, GATA2, GATA2-C295A, and GST-Zf. The boundaries of the Zn finger region were deduced by comparison with chicken GATA1 reported by Boyes *et al.* (1998). The homology of amino acids among GATA1 relative to GATA2 is indicated as a percentage in the boxes. In GST-Zf, GST protein was fused to GATA2-Zf. (B) The transactivation of TSHβ-CAT by GATA1 and GATA2 was suppressed by E_2-ERα. The expression plasmids for mouse GATA1 or 2 (0·4 μg) were transfected together with that for PIT1 (0·1 μg) into CV1 cells. The results are shown as the means ± S.E.M. from six independent experiments. (C) The expression plasmids for mouse GATA2 or GATA2-C295A (0·4 μg) were transfected into CV1 cells under the same conditions as Fig. 1B. The results are shown as the means ± S.E.M. from six independent experiments. The magnitude of the CAT activity stimulated by wild-type GATA2 and PIT1 was taken as 100%. $*P<0·05$; $**P<0·05$ versus reporter alone (first lane). (D) GST-Zf was incubated with ^{35}S-labeled TRβ1 or ERα in the presence or absence of 1 μM T_3 or E_2 respectively. ^{35}S-labeled luciferase protein was utilized as a control. Arrowheads indicate ^{35}S-labeled TRβ1 (upper panel), ERα (middle panel), and luciferase protein (lower panel). luc: luciferase protein. I and B indicate input and bound respectively. (E) ^{35}S-labeled wild-type ERα or its deletion constructs were incubated with GST-Zf. GST-Zf interacted with full-length ERα, Δ170, and N1 but not with Δ247 or N2, which lack DBD. I and B indicate input and bound respectively.

who visited Ito Hospital because of simple goiter or thyroid nodule(s) in May 2004 and none had any thyroid autoantibodies. Because age-related decline in thyroid function is not noticeable until the eighth decade of life (Mariotti *et al.* 1993), we recruited female subjects with ages up to 70 years old as the postmenopausal population. As predicted, the serum E_2 levels in postmenopausal females were very low (Fig. 7, left panel). Although there was no difference in the free T_4 concentrations (middle panel), the serum TSH level of the postmenopausal group was significantly higher than that of the premenopausal group (right panel).

Discussion

In the present study, we demonstrated that E_2-ERα inhibits transactivation of the *TSHβ* gene through a mechanism similar to inhibition by T_3-TRs. We used reporter assay in the CV1 cell line, which is ideal for comparing the mechanism of ligand-dependent negative regulation of the *TSHβ* gene with that of positive regulation (Nakano *et al.* 2004). Our findings imply that redundancy in the recognition of target genes exists not only in the positive regulation (Klinge *et al.* 1997, Scott *et al.* 1997) but also in the negative regulation of the *TSHβ* gene. It was postulated that E_2-dependent inhibition of the

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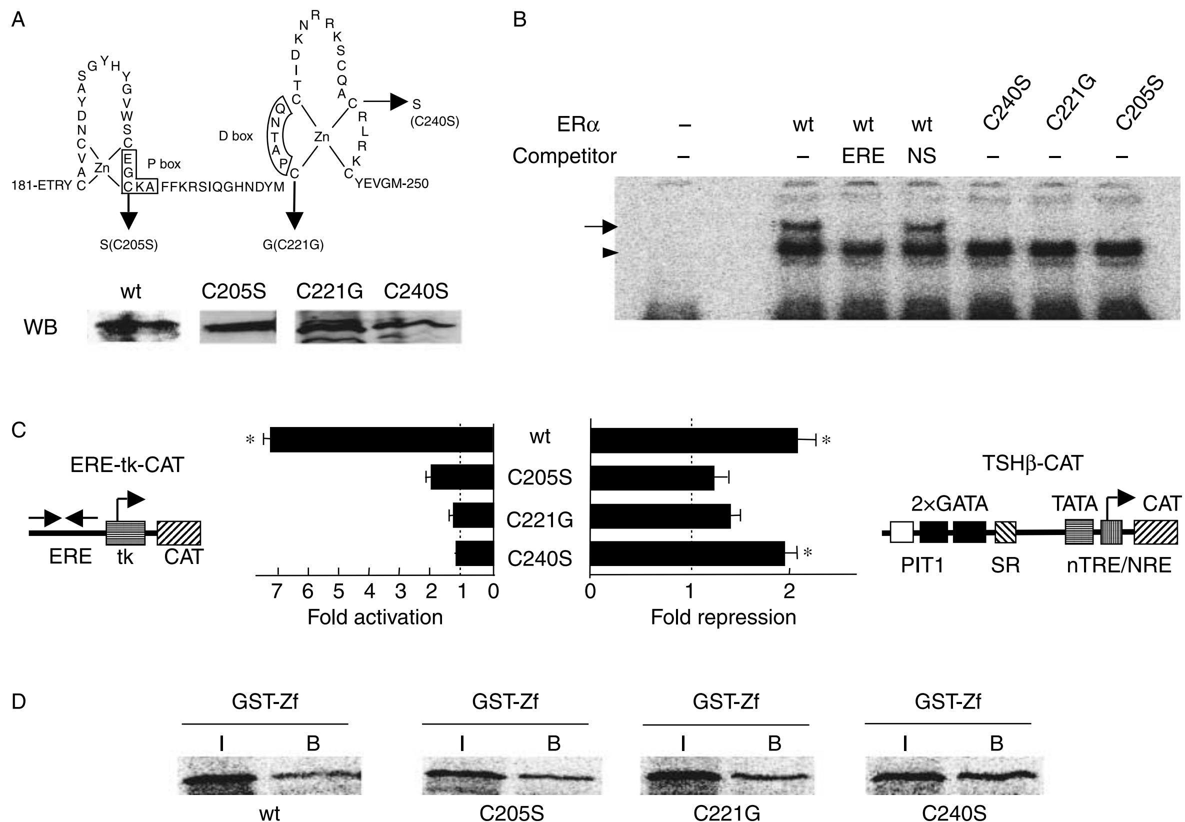

Figure 4 Mutation analysis of ERα-DBD. (A) Schematic of ERα-DBD (upper panel). Cysteines at codon 205 in the P-box and 221 and 240 in the stem of second Zn finger were substituted to serine (C205S), glycine (C221G), and serine (C240S). The expression levels of wild-type and mutant ERαs were comparable (lower panel). CV1 cells were transfected with the expression plasmids for wild-type or mutant ERαs. Whole cell extracts were fractionated by SDS-PAGE and subjected to western blot (WB) with anti-ERα antibody against the C-terminal region of ERα. (B) ^{32}P-radio-labeled vit-A2 ERE was incubated with *in vitro*-translated protein for wild-type or mutant ERαs. ERE, specific cold competitor. NS, non-specific cold oligo DNA. Arrow, specific binding of ERα; arrowhead, nonspecific binding. (C) ERE-tk-CAT (left panel) was transfected into CV1 cells together with the expression plasmid for ERα in the presence or absence of 1 μM E_2. CAT activity with 1 μM E_2 was divided by that without E_2 to calculate fold activation. TSHβ-CAT (right panel) was transfected into CV1 cells together with the expression plasmids for PIT1, GATA2, and wild or mutant ERαs as shown in Fig. 1B. CAT activity without E_2 was divided by that with 1 μM E_2 to calculate fold repression. (D) ^{35}S-radio-labeled receptors were incubated with GST protein fused to GST-Zf and binding fractions were subjected to SDS-PAGE.

TSHβ gene was mediated via direct binding of ERα with the putative nTRE (Wondisford 1996). However, this is unlikely, because E_2-induced inhibition is preserved in the ERα mutant C240S, which fails to bind or activate canonical vit-A2-ERE (Fig. 4C). In agreement with this, E_2-ERα inhibited GATA2-induced transactivation of *TSHβ-D4-CAT*, which lacked a DNA sequence homologous to the half-site. Moreover, E_2-ERα repressed the activity of *TSHβ-M1-CAT* (Fig. 2B) and CGA-CAT stimulated by GATA2 alone (data not shown), suggesting that interference of GATA2-induced transactivation by E_2-ERα is the mechanism for inhibition of the *TSHβ* gene. This notion is further supported by the observation that transactivation by the mutant GATA2-C295A was resistant to E_2-ERα-induced suppression (Fig. 3C). ERα interacts directly with PIT1 (Ying *et al.* 1999), and these two transcription factors synergize in the transactivation of the prolactin gene in lactotrophs (Chuang *et al.* 1997, Schaufele 1999), whose most important PIT1-binding site is located adjacent to the ERE (Nowakowski & Maurer 1994). However, current results indicate that E_2-ERα-dependent inhibition of the *TSHβ* promoter does not require PIT1 (Fig. 2B) and is rather crippled by the overexpression of this transcription factor (Fig. 2A).

Although the results of the transfection experiments as well as the cell culture studies are obtained with high pharmacological doses of E_2, the overall results were in accordance with our recent findings regarding the T_3-dependent inhibition of the *TSHβ* gene (Matsushita *et al.* 2007). The repression of GATA2-induced activation by E_2-ERα and T_3-TR may occur by a 'tethering mechanism' (Nissen & Yamamoto 2000, Herrlich 2001), where liganded nuclear receptors interfere with the activity of DNA-binding transcription factors through protein–protein interactions. In this kind of inhibition, redundancy among liganded

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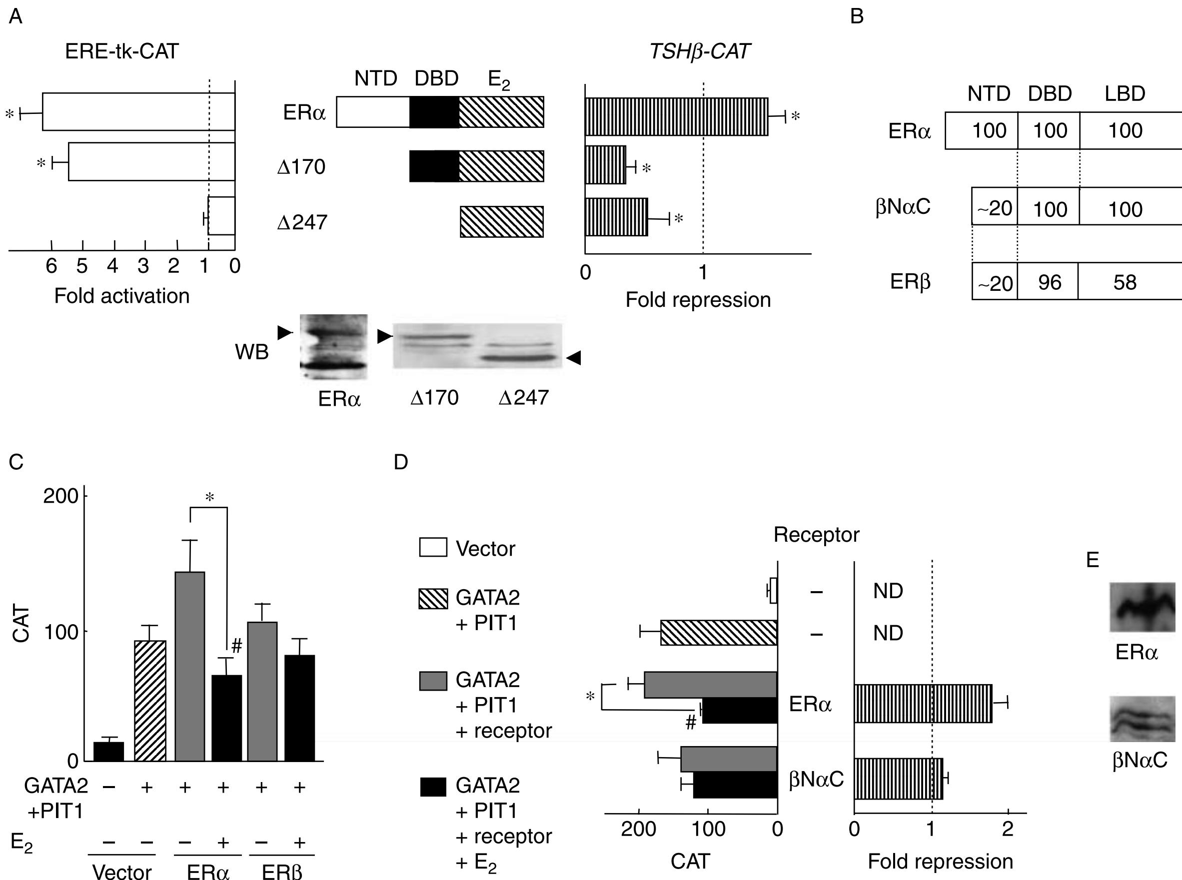

Figure 5 Involvement of NTD in the negative regulation of *TSHβ* gene by E_2-ERα. (A) In the presence of 1 μM E_2, expression plasmids for ERα or its mutants, Δ170 and Δ247 (middle upper panel) were transfected into CV1 cells together with the ERE-tk-CAT reporter gene (left panel) or TSHβ-CAT and the expression plasmids for PIT1 and GATA2 (right panel). $*P<0.05$ versus empty vector for receptor expression plasmids. The CAT activity with the ligand was divided by that without ligand to calculate fold activation (left panels). The CAT activity without ligand was divided by that with 1 μM ligand to calculate fold repression (right panels). The expression levels of wild-type and mutant ERαs were comparable (middle lower panel). The CV1 cells were transfected with the expression plasmid for wild-type or mutant ERαΔ170 and Δ247). Western blot (WB) was performed as Fig. 4A. (B) Schematic of ERα, ERβ and chimeric receptor βNαC. In βNαC, NTD of ERα is replaced with that of ERβ. The homology of amino acids among ERβ and βNαC relative to ERα is indicated as a percentage in the boxes. (C) The expression plasmid for ERα or ERβ was transfected into CV1 cells together with TSHβ-CAT and the expression plasmids for PIT1 and GATA2. $^{\#}P<0.05$ versus PIT1 and GATA2. (D) In the presence or absence of 1 μM E_2, the expression plasmid for ERα or βNαC was transfected into CV1 cells together with TSHβ-CAT and the expression plasmids for PIT1 and GATA2. CAT activity without E_2 was divided by that with E_2 (1 μM) to calculate the fold repression (right panel). The results are shown as means ± S.E.M. for eight separate experiments. $*P<0.05$. (E) Expression levels of wild-type ERα and βNαC were comparable. The CV1 cells were transfected with the expression plasmid for wild-type or βNαC and western blot with antibody against the C-terminal region of ERα was performed as Fig. 4A.

receptors has been reported. For example, E_2-ERα (Kalaitzidis & Gilmore 2005) and glucocorticoid (GC)-bound GC receptor (GR; McKay & Cidlowski 1999, De Bosscher *et al.* 2003) repress transactivation by the proinflammatory transcription factor NF-κB by interacting with it. Similarly, the transcriptional activity of AP-1 (typically the Jun/Fos heterodimer) is impaired by T_3-TR and GC-GR (De Bosscher *et al.* 2003).

Although the precise mechanism of how E_2-ERα inhibits the function of GATA2 in transactivation of the *TSHβ* gene is uncertain, the present analysis provides several insights. First, the GST pull-down assay (Fig. 3E) indicated that DBD is essential for the interaction of ERα with GATA2-Zf as in the case of THRBs (Matsushita *et al.* 2007). Second, E_2-dependent inhibition does not require the tertiary structure of the second Zn finger motif in ERα-DBD, since E_2-dependent inhibition was preserved in the mutant ERα C240S (Fig. 4C), which contains a disrupted second Zn finger motif (Fig. 4A). Third, it should be noted that the mutant ERαs, C205S, and C221G, failed to inhibit E_2-dependent inhibition of the *TSHβ* gene (Fig. 4C), although both mutants can physically bind GATA2-Zf *in vitro*

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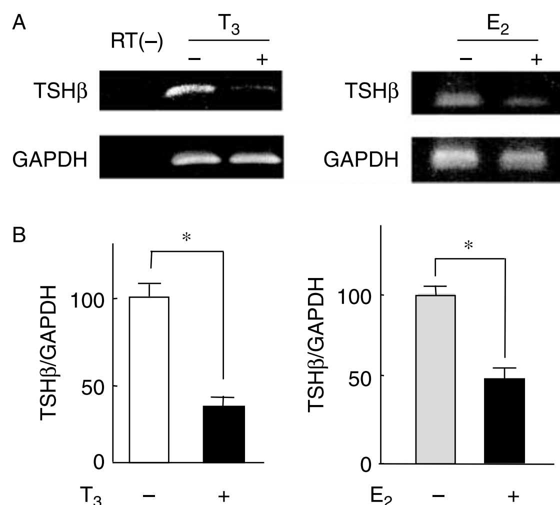

Figure 6 Effect of E_2 on the expression of *TSHβ* mRNA in the cultured thyrotroph cell line, TαT1. (A) *TSHβ* mRNA isolated from TαT1 cells treated with 1 μM T_3 or 1 μM E_2 for 48 h and evaluated with RT-PCR. RT (−), PCR without reverse transcriptase. (B) *TSHβ* mRNA expression evaluated by real-time quantitative RT-PCR. The experiments were repeated five times. The amount of the PCR products was normalized with that of the *GAPDH* gene. The data are shown as means ± S.E.M. Statistical significance was determined by Mann–Whitney *U* test. $^*P<0{\cdot}05$.

(Fig. 4D). The same results were obtained with the mutant TRβ2 G182E (Matsushita *et al.* 2007) and the mutant TRβ1 C127S and C145G (Nakano *et al.* 2004 and data not shown). Physical binding alone is not sufficient to mediate ligand-dependent inhibition, and subtle changes of amino acids on the surface of DBD may be functionally critical. Similar conclusions were made after analysis of mutant GR using circular dichroism (Tao *et al.* 2001).

GATA2-Zf interacts with multiple coactivators including CBP/p300 (Blobel *et al.* 1998, Hayakawa *et al.* 2004),

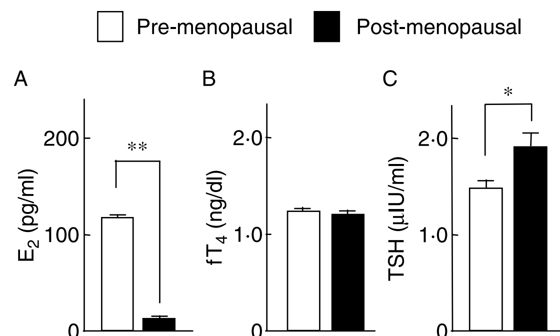

Figure 7 Elevation of serum TSH levels after menopause in women with simple goiter who possess no autoantibodies against thyroglobulin, thyroid peroxidase, or TSH receptor. The levels of E_2 (left panel), free T_4 (middle panel), and TSH (right panel) in 62 premenopausal women and 72 postmenopausal women are indicated. The data are shown as means ± S.E.M. Statistical significance was determined by Mann–Whitney *U* test. $^*P<0{\cdot}05$; $^{**}P<0{\cdot}01$.

TRAP220 (Crawford *et al.* 2002, Gordon *et al.* 2006), and transcriptional repressors such as HDAC3 (Ozawa *et al.* 2001) and FOG 1 and 2 (Ferreira *et al.* 2005). ERα and TR share common coactivators including the p160 family (SRC1, TIF2/GRIP1, and AIB1/ACTR), CBP/p300, p300/CBP-associating factor (PCAF), and TRAP220. As in the case of T_3-THRBs (Matsushita *et al.* 2007), overexpression of SRC-1, CBP, or PCAF did not show reproducible effects on the negative regulation of the *TSHβ* gene by E_2-ERα (data not shown). TRAP220 is required for the expression of the *TSHβ* gene (Ito *et al.* 2000) and functions as a coactivator for GATA2 (Gordon *et al.* 2006), GATA1 (Stumpf *et al.* 2006), TR (Ito & Roeder 2001), and ERα (Zhang *et al.* 2005). TR (Ito & Roeder 2001) and ERα (Zhang *et al.* 2005) recognize an extended amino acid sequence encompassing two LXXLL motifs in TRAP220 with a specificity distinct from that of coactivators in the p160 family (Acevedo & Kraus 2003, Coulthard *et al.* 2003). We reported that dnTRAP220 (Yuan *et al.* 1998) relieved the T_3-TR-dependent inhibition of the TSHβ promoter and that TRAP220 dissociated from this promoter after T_3 treatment of TαT1 cells (Matsushita *et al.* 2007). We found that the expression of the dnTRAP220 also abolished the E_2-ERα-induced inhibition of the *TSHβ* gene (data not shown). Thus, TRAP220 may play a role in the inhibition of the *TSHβ* gene by E_2-ERα and T_3-TR.

We found that ERα-NTD plays an essential role in the repression of the *TSHβ* gene by E_2 (Fig. 5). This domain interacts directly with the p160 family (Lavinsky *et al.* 1998, Webb *et al.* 1998), p300 (Kobayashi *et al.* 2000), HDAC4 (Leong *et al.* 2005), and repressor of tamoxifen transcriptional activity (Norris *et al.* 2002). Although ERα-NTD may bind directly with LBD (Kraus *et al.* 1995, Metivier *et al.* 2000, *et al.* 2001) or regulate the interaction between the antagonist bound LBD and its co-repressor (Lavinsky *et al.* 1998), the βNαC data indicate that ERα-NTD has an intrinsic function to control the E_2-dependent inhibition of the *TSHβ* gene. TRβ2-NTD was reported to have a strong T_3-independent transactivation effect on TRE (Sjoberg & Vennstrom 1995) and also to regulate the interaction between LBD and SMRT (Yang & Privalsky 2001). A function unique to TRβ2-NTD may account for its stronger repression of the *TSHβ* gene than TRβ1 (Nakano *et al.* 2004). Importance of GR-NTD was also reported for ligand-dependent inhibition of AP-1 activity (Heck *et al.* 1994).

E_2 is known to have profound influence on the thyroid hormone system, including cell proliferation (Furlanetto *et al.* 1999), iodine uptake (Furlanetto *et al.* 1999, Alotaibi *et al.* 2006, Lima *et al.* 2006), thyroid peroxidase activity (Lima *et al.* 2006), de-iodinases (Lisboa *et al.* 2001, Wasco *et al.* 2003), or TBG (Gross *et al.* 1971, Surks & Sievert 1995, Arafah 2001). Altered thyroid hormone production and metabolism may mask the direct effect of E_2 on TSHβ expression. To exclude the influences of E_2 on T_3 and T_4 levels in the circulating blood, we measured *TSHβ* mRNA in TαT1 cells and found that E_2 significantly repressed the expression of *TSHβ* mRNA (Fig. 6). We studied serum

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TSH levels in women with simple goiter or thyroid nodule(s), who had no thyroid autoantibodies. The TSH level was significantly higher in the postmenopausal than premenopausal population (Fig. 7). Since the age of the postmenopause group was between 50 and 70 years old, thyroidal deterioration due to aging is unlikely (Mariotti *et al*. 1993). A small but significant increase in the TSH level after menopause may be related to the decreased TSH suppression by E_2. Although free T_4 or serum E_2 data were not available, the NHANES III report (Hollowell *et al*. 2002) also showed that the TSH concentration tended to increase with age in female populations without thyroid autoantibodies. Surks & Hollowell (2007) have recently analyzed TSH frequency distribution curves in NHANES III and showed the progressive shift toward higher concentrations of TSH with age irrespective of thyroid antibodies. They say that an explanation for this shift to higher TSH ranges in elderly people is not apparent, but we consider that our finding in this study is one of the main factors.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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