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Involvement of ETS1 in thioredoxin-binding protein 2 transcription induced by a synthetic retinoid CD437 in human osteosarcoma cells

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ABSTRACT

CD437, a synthetic retinoid, has a potent antitumor activity, in which an RAR-independent mechanism may be involved. Our previous study showed that CD437 transcriptionally upregulates the expression of thioredoxin-binding protein 2 (TBP2), leading to c-Jun N-terminal kinase 1 (JNK1)-mediated apoptosis. In the present study, we addressed the mechanism, by which CD437 induces TBP2 mRNA expression. CD437 efficiently caused the cell death of human osteosarcoma cells via apoptosis. CD437 also induced JNK1 activation through the upregulation of TBP2 mRNA, in consistent with our previous observation. A luciferase reporter assay for TBP2 promoter activation suggested that CD437-regulated TBP2 mRNA transcription requires the region between −400 and −300, which contains multiple possible ETS-binding sites. Finally, we demonstrated CD437-dependent recruitment of ETS1 transcription factor to this region by chromatin immunoprecipitation assay. These data suggest that ETS1 is involved in CD437-induced TBP2 mRNA expression in human osteosarcoma MG-63 cells.

Introduction

Retinoids have diverse physiological properties, including epithelial maintenance, immune defense, embryogenesis and prevention of tumor development [1,2]. These effects are exerted mainly by retinoid's binding to the retinoic acid receptors (RARs) and the retinoid X receptors, leading to the modulation of the transcription of a number of retinoid target genes [1,2]. Recently, retinoid-related molecules with more potent antitumor activity have been developed, one of which CD437 has been shown to inhibit proliferation of various types of malignant cell lines [3]. Although CD437 activates the RAR-dependent pathway [4], it has been reported that RAR-independent pathways are employed, in particular, for CD437-induced apoptosis in cancer cell lines [5]. In accordance with this notion, we previously reported that CD437 induced apoptotic cell death through mitochondrial dysfunction and endoplasmic reticulum stress in retinoic acid resistant cancer cell lines from liver and ovary [6,7]. Moreover, in an ovarian adenocarcinoma cell line, we found that CD437 significantly upregulates thioredoxin-binding protein 2 (TBP2), which mediates the activation of apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase 1 (JNK1) in response to CD437 treatment [8]. Although this result suggested that TBP2 plays an important role in CD437-induced apoptosis, the underlying mechanism of the upregulation of TBP2 transcription by CD437 remains to be clarified.

In this study, we investigated the antitumor action of CD437 in MG-63, an osteosarcoma cell line. Although the effect of this compound on osteosarcoma cell lines had not been reported, MG-63 cells treated with CD437 showed typical apoptotic phenotypes and increased expression of TBP2. In addition, we observed TBP2-dependent JNK1 activation, suggesting that the same mechanism found in the previous study is also employed in the osteosarcoma cell line. Finally, it was revealed for the first time that ETS1 transcription factor plays an important role in CD437-induced TBP2 mRNA expression.

Materials and methods

Cell culture. MG-63 osteosarcoma cell line (RCB1890) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. The cells were cultured in DMEM supplemented with 10% FBS at 37 °C in a 5% CO_2 incubator. Trypan blue exclusion assay, WST assay and Hoechst33258 staining were carried out as previously reported [6].

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DNA ladder formation assay. Apoptotic DNA fragmentation was detected by the method of Herrmann et al. [9]. In brief, 5×10^5 MG-63 cells were seeded on a 10-cm dish and allowed to attach overnight followed by treatment with 0, 1, 2, or 4 μM CD437 (Sigma, St. Louis, MO, USA). After 48 h incubation, the cells were collected by trypsinization and lysed by gentle pipetting in 100 μL of a buffer (50 mM Tris–HCl (pH 7.5), 20 mM EDTA, 1% NP-40). The supernatant was recovered by centrifugation (1600*g*, 5 min) and the extraction was repeated with the same amount of the lysis buffer. The supernatant (200 μL) was digested with proteinase K (final concentration 2.5 μg/μL) at 37 °C for 2 h. After mixing with 100 μL of 10 M ammonium acetate and 300 μL of iso-propanol, DNA was precipitated by centrifugation at 150,000 rpm for 30 min at 4 °C. DNA fragments were separated by 1.2% agarose gel electrophoresis and visualized by ethidium bromide staining.

Small-interfering RNA (siRNA) and gene expression analysis. Transfection of TBP2-specific siRNA (siTBP2), real-time RT-PCR analysis for TBP2 and β-actin, and Western blot analysis for JNK and phospho-JNK were previously reported [8]. Real-time RT-PCR analysis for ETS1 and ESE1 was performed by using primers (ETS1 sense, 5′-ATCAAACAAGAAGTCGTCACCC-3′; anti-sense, 5′-CAACTATCGTAGCTCTCTATGCTTTCA-3′; ESE1 sense, 5′-CACTCCTCAGACTCCGGT-3′; anti-sense, 5′-CCCTTCTTGCAGTCACGAA-3′).

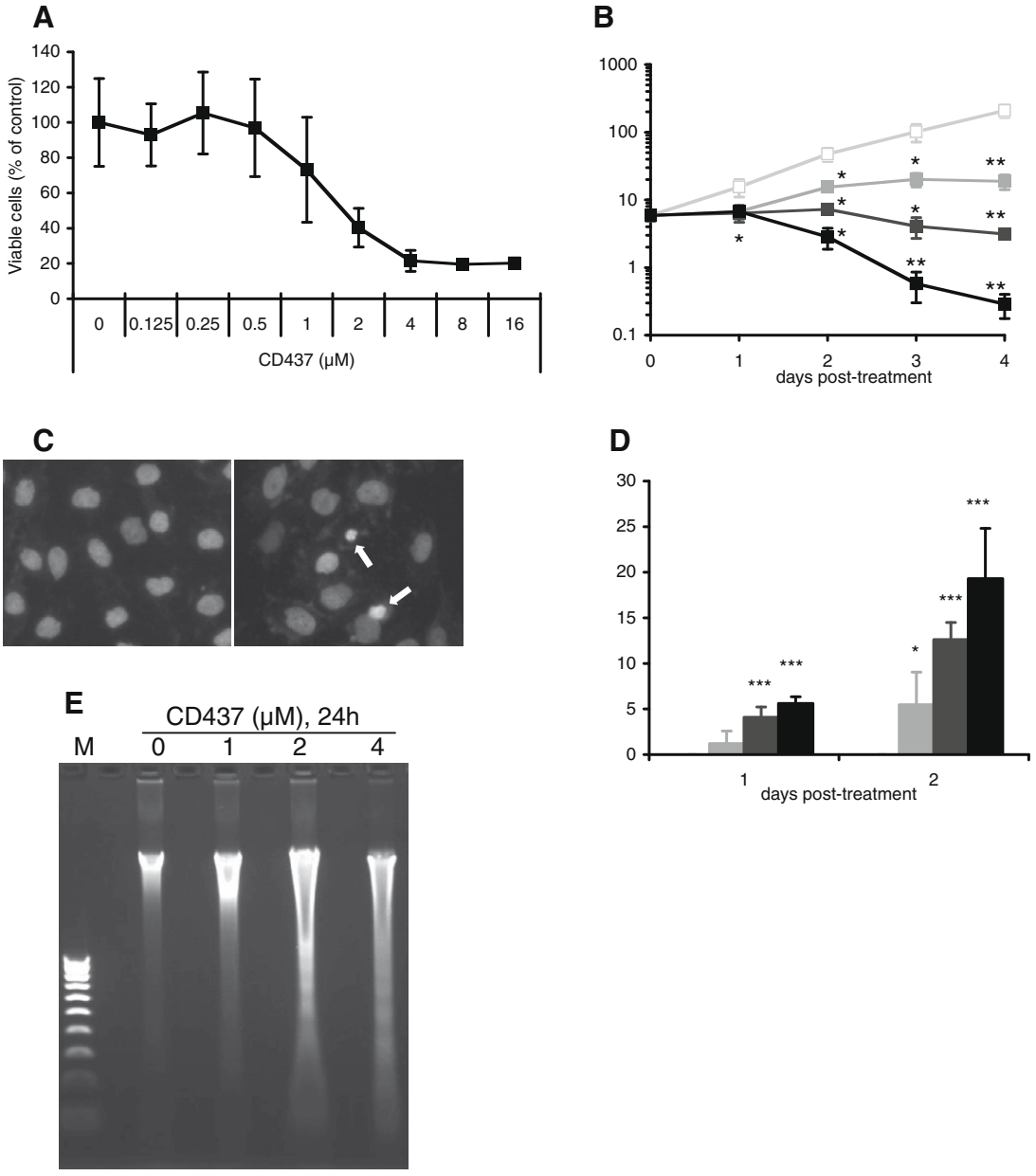

Fig. 1. CD437-induced apoptosis in MG-63 cells. (A) Cell viability in the presence of CD437. WST assay was performed following 3-day treatment of MG-63 cells with the indicated concentrations of CD437. Data are shown as means ± SD ($n = 3$). (B) Kill curve for MG-63 cells treated with CD437. MG-63 cells were incubated with 0 (open squares), 1 (light gray squares), 2 (gray squares) or 4 μM (black squares) of CD437 for the indicated periods. The number of cells was determined by trypan blue exclusion assay. Data are shown as means ± SD ($n = 4$). Statistical significance was determined by Student's *t* test: $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control. (C) Apoptosis-specific nuclear morphology. MG-63 cells cultured for 24 h in the presence of 0 (left) or 4 (right) μM of CD437 were further incubated with 10 μM of Hoechst33258 for 1 h. (D) The increased fraction of apoptotic cells. After MG-63 cells were treated with 0 (open bars), 1 (light gray bars), 2 (gray bars) or 4 μM (black bars) of CD437 for the indicated periods. The number of cells with apoptosis-specific nuclear morphology was counted and their ratio was calculated as previously reported [6]. Statistical significance was determined by Student's *t* test: $^{*}P < 0.05$; $^{***}P < 0.001$ vs. control. (E) Apoptosis-specific interchromosomal DNA fragmentation. DNA ladder formation assay was performed after 24-h treatment of MG-63 cells with the indicated concentration of CD437. M, 100-bp marker.

Luciferase reporter gene assay. Various lengths of TBP2 promoter regions including 5′-untranslated region (nucleotides position; −3k, −2k, −1k, −500, −400, −300, −200, −100, and +1 to +220 relative to the transcription start site defined in GenBank Accession No. NM_00647.2) were amplified using KOD Plus (TOYOBO, Osaka, Japan), and were cloned into XhoI–HindIII site of pGL3-basic (Promega, Madison, WI). ETS transcription factor expression vectors were previously reported [10,11]. Transfection complex was prepared by mixing 0.4 μg TBP2 promoter vector, 0.2 μg phRL-TK (Promega) and 0.9 μL FuGene6 (Roche, Tokyo, Japan) or by mixing 0.5 μg ETS transcription factor expression vectors, 0.4 μg TBP2 promoter vector, 0.2 μg phRL-TK and 1.5 μL FuGene6 in 200 μL Opti-MEM (Invitrogen, Carlsbad, CA). MG-63 cells (8×10^3) were seeded on a 24-well plate and allowed to attach overnight. The cells were incubated with the transfection complex for 36 h followed by treatment with 0 or 4 μM CD437 for 12 h. The luciferase activities were determined by the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instruction.

Chromatin immunoprecipitation (ChIP) assay. MG-63 cells were seeded on 10-cm dishes at 5×10^5 cells per dish and allowed to attach overnight followed by treatment with 0 or 4 μM CD437 for 12 h. The cells were fixed with 1% formaldehyde for 10 min at room temperature, and were subsequently incubated with 125 mM glycine for 5 min at room temperature. After washing the cells with cold phosphate buffered saline (PBS) twice, the cells were scraped, counted, and collected by centrifugation (1000*g*) for 5 min. The cell pellets were lysed in 100 μL of sodium dodecyl sulfate (SDS)–lysis buffer (50 mM Tris–HCl (pH 8.0), 1 mM EDTA and 1% SDS) and 4 μL of 25× protein inhibitor cocktail solution (PIC; Roche) per 10^6 cells for 10 min on ice, and then sonicated 10 times for 30 s (interval 30 s) on ice. The supernatant was recovered by centrifugation (150,000 rpm, 10 min, 4 °C). Subsequently, 100 μL of the supernatant was mixed with 900 μL of dilution buffer (16.7 mM Tris–HCl (pH 8.0), 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100), 40 μL of 25× PIC, 50 μL (10 μg) of ETS1 rabbit polyclonal antibody (C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and 25 μL of protein G magnetic beads (Active Motif, Carlsbad, CA) in a siliconized tube, and the mixture was rotated for 36 h at 4 °C. The beads were washed each two times with 1400 μL of Low Buffer (20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100), High Buffer (20 mM Tris–HCl (pH 8.0), 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100), LiCl Buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 250 mM LiCl, 1% sodium deoxycholate, 1% NP-40), and TE Buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA). Finally, the beads were incubated with 12.5 μL of elution buffer (0.1 M $NaHCO_3$, 10 mM DTT, 1% SDS) for 15 min at room temperature, and the elution was repeated with the same amount of elution buffer. After adding 10 μL of 5 M NaCl and 4 μL of 20 mg/mL proteinase K, the eluates were reverse cross-linked by heating at 65 °C at least 4 h. The DNA fragment was purified by QIAquick PCR purification kit (QIAGEN, Hilden, Germany). Real-time PCR analysis was performed by a LightCycler System (Roche) using primers specific for TBP2 promoter region (forward, 5′-TCGCCCTGGCTAGGTTT-3′; reverse, 5′-ATGGTTGTTGCGCTCTGGA-3′: Primer positions are indicated in Fig. 3A) and +6k down stream region of TBP2 gene (forward, 5′-CCCTTAGATGATTTGATGGTTCCTATTC-3′; reverse, 5′-CTGGGAGACA GAGCAAGACT-3′).

Results

Apoptosis induction of MG-63 cells by CD437

CD437 efficiently inhibited MG-63 cell growth, and decreased the number of viable cells in a dose-dependent manner (Fig. 1A and B). The cells treated with CD437 exhibited apoptosis-specific nuclear morphology (Fig. 1C), and the number of apoptotic cells was increased in dose- and time-dependent manners (Fig. 1D). Moreover, interchromosomal DNA fragmentation, a whole mark for apoptosis, was apparently observed after 24-h treatment with

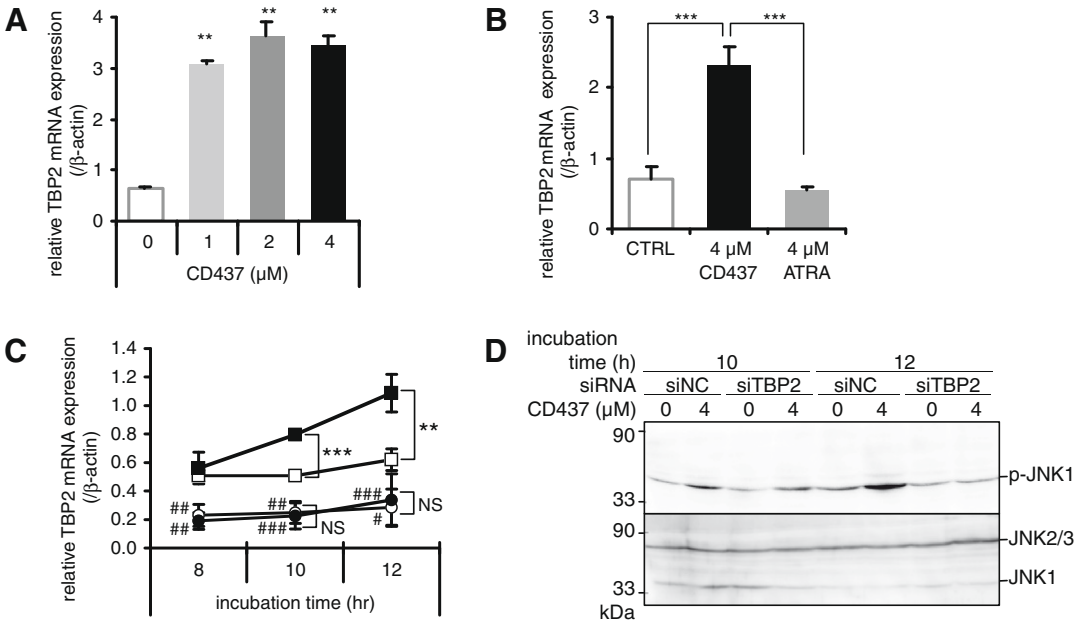

Fig. 2. CD437-induced upregulation of TBP2 expression. (A, B) Real-time RT-PCR analysis of TBP2 mRNA expression. MG-63 cells were treated with 0 (open bar), 1 (light gray bar), 2 (gray bar) or 4 μM (black bar) of CD437 for 12 h (A), or with DMSO (CTRL; open bar), 4 μM CD437 (black bar) or 4 μM ATRA (gray bar) for 12 h (B). TBP2 mRNA levels were normalized by those of β-actin. Data are shown as means ± SD ($n = 3$). Statistical significance was determined by Student's *t* test: $^{**}P < 0.01$; $^{***}P < 0.001$ vs. control and ATRA. (C) SiRNA-mediated knockdown of TBP2. After 36-h transfection with negative control siRNA (siNC; squares) or TBP2-specific siRNA (siTBP2; circles), MG-63 cells were treated with 0 (open symbols) or 4 (filled symbols) μM CD437 for the indicated periods. Data are shown as means ± SD ($n = 3$). Statistical significance was determined by Student's *t* test: NS, not significant; $^{**}P < 0.01$; $^{***}P < 0.001$ vs. 0 μM CD437. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$ vs. siNC. (D) Western blot analysis for JNK phosphorylation. The total proteins were recovered from MG-63 cells treated with 0 or 4 μM CD437 for the indicated periods following transfection with siRNAs.

2 and 4 μM CD437, respectively (Fig. 1E). Thus, these results suggest that CD437 induces cell death via apoptosis in MG-63 cells.

CD437-induced upregulation of TBP2

Similar to the previous observation in an ovarian adenocarcinoma cell line [8], CD437 strongly induced the expression of TBP2 mRNA expression (Fig. 2A). However, no upregulation was observed with a natural RAR ligand all-*trans*-retinoic acid (ATRA) (Fig. 2B), suggesting that an RAR-independent mechanism is involved in the CD437-induced transcriptional regulation of TBP2.

Our previous report demonstrated that, in an ovarian adenocarcinoma cell line, the upregulation of TBP2 expression is required for ASK1-mediated activation of JNK1 upon apoptosis induction by CD437 [8]. Transfection of MG-63 cells with TBP2-specific siRNA (siTBP2) downregulated TBP2 expression even in the presence of CD437 (Fig. 2C). It was observed that CD437 activated JNK1 after 10- and 12-h treatments in the cells transfected with negative control siRNA (siNC) while no significant activation of JNK1 was observed in the cells transfected with siTBP2, especially at 12-h treatment (Fig. 2D). These results suggest that apoptosis in MG-63 cells after CD437 treatment is JNK1-mediated, which is similar to the previous observation in an ovarian adenocarcinoma [8].

Involvement of ETS transcription factors in TBP2 expression

To clarify a CD437-response element in the TBP2 promoter, various lengths of TBP2 promoter regions including 5′-untranslated region were inserted into the upstream of luciferase reporter gene (Fig. 3A). The reporter gene assay revealed that a region from −400 to −300 is critical for response to the treatment of CD437 (Fig. 3B). Surprisingly, the concatemerized ETS-binding sites (EBS: [12])

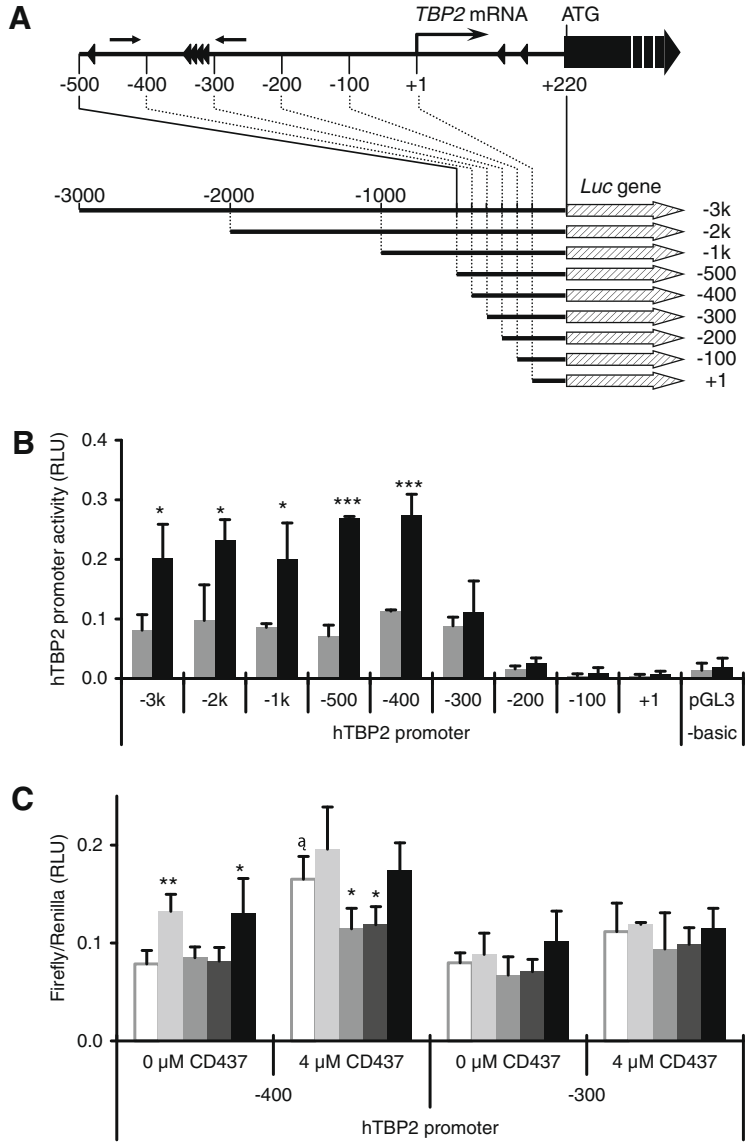

Fig. 3. Luciferase assay for TBP2 promoter activation. (A) The map of luciferase reporter constructs. Triangles indicate putative ETS-binding sites predicted by TESS [13]. Paired arrows indicate the location of ChIP primers used in Fig. 4B. (B) TBP2 promoter activities following 12-h treatment with 0 (gray bars) or 4 (black bars) μM CD437 were determined by luciferase assay. pGL3-basic was used as promoterless control reporter plasmid. Data are shown as means ± SD ($n = 4$). Statistical significance was determined by Student's t test: $^{*}P < 0.05$; $^{***}P < 0.001$ vs. 0 μM CD437. (C) TBP2 promoter activities following 12-h treatment with 0 or 4 μM CD437 in MG-63 cells co-transfected with EGFP (negative control; open bars), ETS1 (light gray bars), ELF1 (gray bars), TEL (dark gray bars) and ESE1 (black bars) expression vectors were determined by luciferase assay. Data are shown as means ± SD ($n = 4$). Statistical significance was determined by Student's t test: $^{*}P < 0.05$; $^{**}P < 0.01$ vs. EGFP expression vector. $^{\dagger}P < 0.05$; $^{\ddagger}P < 0.001$ vs. 0 μM CD437.

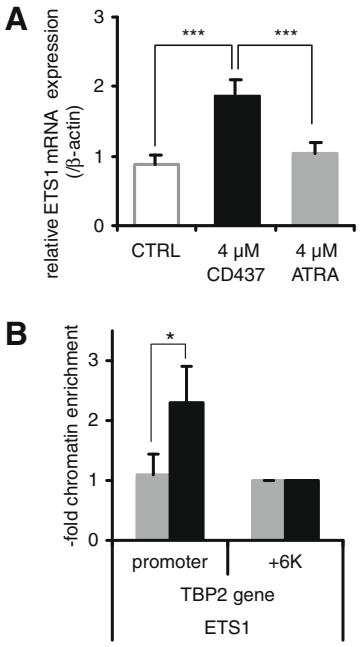

Fig. 4. Involvement of endogenous ETS1 in transcriptional induction of TBP2 by CD437. (A) Real-time RT-PCR analysis of ETS1 mRNA expression. MG-63 cells were treated with DMSO (CTRL; open bar), 4 μM CD437 (black bar) or 4 μM ATRA (gray bar) for 12 h. ETS1 mRNA levels were normalized by those of β-actin. Data are shown as means ± SD ($n = 3$). Statistical significance was determined by Student's t test: $^{***}P < 0.001$. (B) ChIP analysis for ETS1 recruitment to TBP2 promoter. MG-63 cells were treated with 0 (gray bars) or 4 (black bars) μM CD437 for 12 h, and then ChIP assay was performed using ETS1 antibody and primers indicated in (A). Data are shown as means ± SD ($n = 4$). Statistical significance was determined by Student's t test: $^{*}P < 0.05$.

were predicted in this region by TESS [13] while the EBS was rarely found outside this region (Fig. 3A). Thus, the reporter gene assay was performed following the co-transfection of the reporter vectors containing TBP2 promoter region from −400 or −300 to +220 with ETS transcription factor expression vectors (ETS1, ELF1, TEL and ESE1) or a control EGFP expression vector. It was found that ETS1 and ESE1 increased TBP2 promoter activity in the absence of CD437 while no effect was observed by ELF1 and TEL (Fig. 3C). Moreover, in the presence of 4 μM CD437, ETS1 further increased TBP2 promoter activity while ESE1 did not affect its activity compared to that in the absence of CD437 (Fig. 3C). Of interest, the co-transfection of ELF1 and TEL significantly impaired the activation of TBP2 promoter by CD437 (Fig. 3C), implying that these exogenous ETS transcription factors may compete with endogenous ETS transcription factors for the same EBS. These results suggest that TBP2 promoter region from −400 to −300 is responsive site to ETS transcription factors, particularly ETS1 and ESE1.

Involvement of endogenous ETS1 in CD437-induced regulation of TBP2 expression

Finally, we explored an endogenous transcription factor responsible for CD437-induced regulation of TBP2 expression. ETS1 expression was significantly upregulated by CD437 but not by ATRA (Fig. 4A) while no expression of ESE1 was detected in MG-63 cells (data not shown). Furthermore, using anti-ETS1 antibody, we performed ChIP assay, in which enriched DNA was quantified by real-time PCR using primers specific for TBP2 promoter region (−424 to −282; position is indicated in Fig. 3A) and +6k down stream region of TBP2 gene. As shown in Fig. 4B, CD437-dependent recruitment of ETS1 to the promoter region was clearly observed. These results suggest that ETS1 plays an important role in CD437-induced upregulation of TBP2 expression.

Discussion

In the present study, we demonstrated for the first time the involvement of the ETS1 transcription factor in the mechanism of CD437 action. The ETS family is a large transcription factor family with more than 30 members, all of which share the conserved winged helix-turn-helix DNA-binding domain, termed the ETS domain [12,14]. The EBS comprising GGAA/T is recognized as the core-binding site for ETS proteins [12]. As the EBS is found in promoter and enhancer regions of numerous genes, ETS proteins are involved in diverse physiological functions, such as cellular proliferation, differentiation, senescence and death, and tumorigenesis. ETS1, a cellular counterpart of the v-*ets* oncogene product of the avian erythroblastosis virus E26, acts as a proto-oncogene since its overexpression induced transformation of fibroblast cells and rendered the cells tumorigenic in nude mice [15]. Moreover, rearrangement and amplification of *ETS1* locus in leukemia and lymphoma [16] and elevated ETS1 expression in a various malignant tissues including breast, lung, colon, pancreatic and thyroid cancers [12] had been found. On the other hand, ETS1 is suggested to possess anti-oncogenic properties. Ohtani et al. observed significant upregulation of ETS1 in senescent fibroblasts with increased $p16^{INK4a}$ expression, but not in young cells, in which ETS2 is abundant [17]. The study also found that ETS1 plays a direct and positive role in senescence-associated induction of $p16^{INK4a}$ expression in the primary fibroblasts [17]. Moreover, it was shown that ETS1 sensitized DLD-1 colon carcinoma cell line to Fas-induced apoptosis through the upregulation of caspase-1, whose promoter region contains functional EBS [18]. Our study apparently demonstrated the aspect of ETS1 as an anti-oncogene because of the finding that ETS1 is involved in the transcriptional regulation of *TBP2* gene.

TBP2 is known to bind to and suppress thioredoxin (TRX) activity while TRX has an anti-apoptotic effect by inhibiting ASK1. We previously reported that CD437 induced the association of TBP2 with TRX, and in turn facilitated the dissociation of ASK1 from TRX in an ovarian adenocarcinoma cell line [8]. Moreover, the activation of ASK1 and its downstream molecule, JNK1, was observed after TBP2 induction by CD437, leading to cell death via apoptosis. In addition to ASK1 and JNK1 activations, it has been reported that ectopic expression of TBP2 suppressed cell proliferation by arresting the cell cycle at G1 phase in association with an increase of $p16^{INK4a}$ expression and reduction of retinoblastoma phosphorylation [19]. Although the precise mechanism remains to be clarified, it is possible that senescence is involved in the mechanism of CD437 action.

Taken together, this study revealed that ETS1 is the transcriptional machinery of TBP2 expression upon CD437-induced apoptosis. Further studies on the molecular mechanisms leading to ETS1 activation by CD437 will provide valuable insights into the molecular basis of carcinogenesis and the development of more effective anti-cancer therapies.

Conflict of Interest disclosure

The authors declare no competing financial interests.

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