Abstract

Ceramide is the central lipid in the sphingolipid metabolism. Ceramide kinase (CERK) and its product, ceramide 1-phosphate (C1P), have been implicated in various cellular functions. However, the regulatory mechanism of *CERK* gene expression remains to be determined. Here, we examined *CERK* mRNA level during all-*trans* retinoic acid (ATRA)-induced differentiation of a human neuroblastoma cell line, SH-SY5Y. ATRA reduced CERK mRNA and protein levels. Overexpression and siRNA of *CERK* revealed that CERK is inhibitory against ATRA-induced neuronal differentiation and cell growth arrest. ATRA inhibited the transcriptional activity of 5'-promoter of *CERK*. Truncation and mutation study suggests that ATRA-responsible region was mainly located in the tandem retinoic acid responsive elements (RARE) between -40 bp and the first exon. The electrophoresis mobility shift assay revealed that ATRA produced two retarded bands, which were erased by antibody against COUP-TFI, RARα and RXRα, respectively. DNA pull-down assay confirmed increased binding of these transcription factors to RARE. Transient expression of *RAR*, *RXR* and *COUP-TFI* and siRNA transfection of these genes revealed that COUP-TFI inhibited CERK mRNA. Furthermore, chromatin immunoprecipitation assay showed the recruitment of co-repressors as well as three transcription factors. These results suggest that COUP-TFI was the ATRA-responsive suppressive transcription factor of CERK gene transcription.

Keywords: All-*trans* retinoic acid, neuronal differentiation, SH-SY5Y cell, ceramide kinase, promoter analysis, COUP-TFI.

Introduction

Ceramide is the central core lipid in the metabolism of sphingolipids. It is produced either from de novo synthesis starting by the enzyme serine palmitoyl-CoA transferase, the hydrolysis of sphingomyelin by sphingomyelinase or through the salvage pathway from complex sphingolipids[1,2,3]. It can be further metabolized to sphingosine and sphingosine 1-phosphate (S1P), or synthesized to sphingomyelin and glycosphingolipid. It can also be phosphorylated to ceramide 1-phosphate (C1P) by ceramide kinase (CERK) [1,4].

The CERK activity was first described in brain synaptic vesicles[5]. It can be also found in HL60 cells and neutrophils[6,7]. Based on the sequence homology to sphingosine kinase 1 (SPHK1), CERK was cloned[8]. CERK mRNA expression is high in brain, heart, skeletal muscle, kidney and liver[8]. CERK is a highly conserved lipid kinase in both animals and plants. Concerning the balance of sphingolipid metabolites, it has been proposed that the sphingolipid rheostat model explains the cell's fate by the cellular balance of ceramide and sphingosine/S1P[9]. In addition to S1P, which is well characterized as the intra- or intercellular messenger, C1P is another phosphorylated bioactive sphingolipid[1]. Recent studies have revealed the involvement of CERK in inflammatory process[10], phagocytosis[6,11], cell proliferation and cell survival[12,13]. Previous reports mostly suggested that CERK is cytoprotective and functions to convert pro-apoptotic ceramide to pro-survival C1P depending on the expression level[1,14].

Several groups analyzed the mechanism of CERK enzyme activation. Some investigators recently reported that calmodulin is involved in the calcium-dependent activation of CERK, acting as a calcium sensor for the enzyme[15]. Cardiolipin has been recognized as a cofactor for CERK[5], however, the mechanism of CERK transcription has not been analyzed fully. The use of chromatin immunoprecipitation followed by DNA chip assay (ChIP on Chip) revealed that *CERK* is upregulated in response to phosphorylation of the cyclic AMP response element-binding protein (CREB) and identifies a CREB binding site on the promoter[16]. However, the physiological significance of these results is not known at present. Although preliminary data on the sequential change of *CERK* mRNA during macrophage-like but not granulocytic differentiation of HL60 cells have been reported[17], further information is necessary to understand the physiological meaning of CERK in living cells.

To our knowledge, this is the first study to report the decrease of *CERK* mRNA during ATRA-induced neuronal differentiation of a human neuroblastoma cell line, SH-SY5Y. The effect of ATRA on both cell proliferation and differentiation was modulated with the overexpression of *CERK* and siRNA of *CERK*, suggesting the important role of CERK as the antagonist of ATRA-induced neuronal differentiation and neuronal cell growth arrest. We further analyzed the molecular mechanism of ATRA-induced inhibition of CERK transcription, and found that COUP-TFI, an ATRA-inducible transcription factor, is the main regulator of *CERK* gene expression with ATRA. Since the brain is a rich source of CERK, the significance of CERK during the neuronal differentiation process was discussed.

Materials and methods

Cell line and reagents

A human neuroblastoma cell line, SH-SY5Y, was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All-*trans* retinoic acid and All-*trans* retinol were purchased from Sigma Aldrich (St. Louis, MO, USA). Methoprene acid, a specific agonist of RXRα, was obtained from Biomol (Plymouth Meeting, PA, USA). For siRNA experiments, siRNA of human *CERK* (5'-UGCCUGCUCUGUGCCUGUA-3')[18], *COUP-TF1* (5'-AAGCACUACGGCCAAUUCACC-3')[19], *RARα* (5'-GAACAUGGUGUACACGUGU-3'), *RXRα* (5'-AGGUGAAAGCUUCGUCCGAGA-3') and scrambled siRNA (5'-UAGCGACUAAACACAUCAA-3') were purchased from Sigma Genosys (Hokkaido, Japan). Transfection was performed as described previously[20].

Cell proliferation and cell death

SH-SY5Y cells (5000 cells/well) were cultured in a 96-well plate with or without All-*trans* retinoic acid (ATRA) (10 μM). Cell proliferation was determined in triplicate with WST-1 assay kit (Roche Applied Science, Mannheim, Germany). The initial cell density was determined as 1.0. For the examination of cell death, SH-SY5Y cells were cultured in a 96-well plate for 24 h, and the culture medium was replaced with serum free medium with or without ATRA. Supernatants were collected after 1, 2, 4, and 6 days, and measured LDH activity with LDH-cytotoxic test (Wako Pure Chemical Industries, Osaka, Japan). 100% cell death was determined when total cells in wells were dead. Viable cell number was counted with trypan-blue dye exclusion method.

Quantification of neurite formation and cell number

Cells with neurites longer than 50 μm were counted as neurite-positive under an inverted microscope. At least 200 cells were counted per sample. Experiments were repeated at least three times. The mean +/- SD was calculated.

Ceramide kinase (CERK) assay

CERK activity was determined as described previously[5] with some modification. Briefly, cells were lyzed in a buffer containing 10 mM HEPES, 1 mM DTT and Complete™ protease inhibitor mixture (Roche Applied Science). The lysate was incubated for 60 min at 37℃ in 50 μl reaction mixture containing 0.1 M Tris-HCl (pH 7.4), 80 mM KCl, 3 mM CaCl2, 1 mM cardiolipin, 0.15% Triton X-100, 0.2 mM diethylenetriamine pentaacetic acid, 10 mM ATP, 1mM DTT, 5 mM MgCl2 and 36 μM ^{3}H-oleoyl ceramide (American Radiolabeled Chemicals, MO, USA). Lipids were extracted and separated on Silica Gel 60 high-performance TLC plate (HPTLC, Merck, Darmstadt, Germany) using chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1,v/v) as the developing solvent. The radioactive band corresponding to ceramide-1-phosphate was quantified using an imaging analyzer BAS2500 (Fuji Film Co., Tokyo, Japan).

Establishment of CERK stable transfectants

Human *ceramide kinase (CERK)* cDNA was the kind gift of Dr. T. Kohama (Pharmacology and Molecular Biology Research Laboratories, Daiichi-Sankyo Co., Ltd., Tokyo, Japan.). For inserting *CERK* cDNA into pcDNA3.1 (+) expression vector (Invitrogen, Carlsbad, CA,

USA), PCR were performed using the following primers. Forward: 5'-GGGGAATTCAATGGGGGCGACGGGG-3' (underline indicates *Eco*RI site), Reverse: 5'-CCTTTTCCTCCAAACGGGTTGATA-3'. PCR conditions were 40 cycles of 15 s at 96°C followed by 30 s at 54°C, and 1 min at 68°C. PCR product was digested by *Eco*RI and *Pst*I. *Pst*I site was present inside of the PCR product. Digested product was inserted into pcDNA3.1 (+), which was digested by *Eco*RI and *Xba*I. This construct was named as pcDNA3.1/hCERK.

To establish stable transfectants of *CERK*, DNA transfection was performed by the calcium phosphate precipitation method. Briefly, 20 μg of pcDNA3.1/hCERK expression vector was transfected to SH-SY5Y cells for 6 h. After glycerol shock, cells were cultured in DMEM supplemented with 10% FBS. G418 selection was started after 2 days at the concentration of 400 μg/ml. After limiting dilution, established clones were checked by Western blotting using anti-CERK antibody (ABGENT, San Diego, USA). Two subclones (sc 17 and 11) showing the highest expression of CERK were used for further experiments.

Western blotting

Western blotting was performed as described previously[20]. Antibodies used for Western blotting were anti-CERK antibody (ABGENT San Diego, USA), anti-growth associated protein-43 (GAP-43) antibody (H-100, Santa Cruz Biotechnology, CA, USA), anti-chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) (H-60, Santa Cruz), anti-RARα antibody (C-20, Santa Cruz), anti-RXRα antibody (D-20, Santa Cruz) and anti-β-actin antibody (Cytoskeleton Inc. Denver, CO, USA), respectively.

Transient transfection followed by immunoprecipitation

The plasmids of *RARα, RXRα,* and *COUP-TFI* were the gift of Dr. W. H. Miller (McGill University, Quebec, Canada), Dr. R. M. Evans (The Salk Institute for Biological Studies, CA, USA), and Dr. G. Salbert (Universite de Rennes I, Rennes, France), respectively. After transfection with respective plasmid DNA using lipofectin reagent (Invitrogen), SH-SY5Y cells were treated with either ATRA or methoprene acid for 24 h. Immunoprecipitation assay was performed as described previously[21] with some modification. Cells were washed in PBS, and dissolved in TEN-modified buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% NP-40, 5 mM EDTA, protease inhibitor). Lysates were incubated with protein G PLUS-agarose beads (Santa Cruz) linked with anti-RXRα antibody (F-1, sc-46659, Santa Cruz) or Exacta Cruz™ F beads kit: (sc-45043, Santa Cruz) which were linked with anti-chicken ovalbumin upstream promoter transcription factor I (COUP-TFI) antibody (H-60, Santa Cruz) at 4°C overnight. Beads were washed four times with TEN-modified buffer, and the immune complexes were released from the beads by boiling in SDS-sample buffer for 5 min. Immunoprecipitates were analyzed by Western blotting using either anti-RARα or anti-COUP-TFI antibody.

Quantitative RT-PCR

RNA was isolated using RNeasy Mini kit (QIAGEN, Germany), and the first strand cDNA was prepared with 5 μg of RNA using the Super Script III First-Strand System (Invitrogen). Quantitative RT-PCR was performed with Power SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) in triplicate as described previously[22].The primers for *CERK* forward 5'-AGTCCACCACAACAGCAC-3', reverse 5'-GAGGAAGGTCTTTAAACCTG-3', *SPHK1* forward 5'-TCCTGGCACTGCTGCACTC-3', reverse 5'-TAACCATCAATTCCCCATCCAC-3', and

GAPDH forward 5'-CAGGAGCGAGATCCCTCCAA-3', reverse 5'-CCCCCTGCAAATGAGCCC-3'. PCR conditions were 40 cycles of 15 s at 95ºC followed by 1 min at 54ºC. The standard curve was created using a cloning vector containing the *CERK*-, and *GAPDH*-PCR product, respectively. Incubation and on-line detection of PCR products were carried out with optical 96-well reaction plates in the ABI PRISM 7000 sequence detection systems (Applied Biosystems). Expression level was calculated as the ratio of *CERK*/*GAPDH*.

Rapid amplification of 5'cDNA ends (5'RACE)

RNA ligase-mediated rapid amplification of 5'-cDNA ends (5'-RACE) was performed with a Gene Racer kit (Invitrogen) to determine the transcription initiation site of *CERK* in SH-SY5Y cells. The reverse *CERK* gene-specific primer and reverse *CERK* gene-specific nested primer were 5'-CATTTTCTGCCATTTTCCACTGCCTT-3', and 5'-GTCTGTTTCCTCAACGGCGATGATCT-3', respectively. Cloned PCR products were analyzed for their DNA sequences, and the 5'-transcription initiation point was determined. 5'-transcription initiation point used in the present study was shown in Figure 4.

Cloning of 5'-promoter of human CERK and Luciferase assay

According to DNA sequence of 2.5 kb 5'-promoter region of human *CERK* (DBTSS:http://dbtss.hgc.jp/), the following primer set were prepared for PCR. Forward: 5'-TCCAGTGCTGTGCCCAGAGTCATGG-3', reverse: 5'-AGGCTGGGGGCGCGCGGA-3'. The PCR product was cloned to pBluescript IIKS(+) making the pBluescript IIKS(+)/-2.5 kb promoter. Then, the construct digested by *Kpn*I, and a pGL3 basic vector (Promega) digested by *Kpn*I and *Sma*I were ligated together. This final construct was named pGL3/-2.5 kb promoter. Deletion mutants of the 5'-promoter region of human CERK were prepared using Exonuclease III. pGL3/-2.5 kb promoter was digested by *Kpn*I and *Hin*dIII, and Exonuclease III was added for further digestion. Reaction time was 120, 210, 270, 280, and 360 s. Digested product was self-ligated. As a result, pGL3/-1.7, -1.1, -0.7, -0.6, -0.17 kb were prepared. To circumvent putative transcriptional factor binding sites, further deletion mutants were prepared by PCR-based method and digested by restriction enzymes.

Primers were as follows. For -100 bp promoter, 5-GGGGGTACCCTCCCGGCCCCTCCTCTA-3': for -85 bp promoter. 5-GGGGGTACCCTACCCACGCCTCCTCCTC-3': for -28 bp promoter, 5-GGGGGTACCGCGTACGGGGTGACGCAG-3': for -3 bp promoter, 5-GGGGGTACCCCTCCCGCCGCCCGCC-3' (underline indicates *Kpn*I site). Both PCR product and pGL3 basic vector were digested by *Kpn*I and *Nco*I, and were ligated. pGL3/-40 bp promoter was prepared by digesting pGL3/-2.5 kb with *Kpn*I and *Sac*II following blunting and self-ligation.

To introduce the mutation to the DNA sequence of retinoic acid responsive element, the following primer set were prepared. Forward, 5'-GGGGGTACCGGAAATTTCAAATTTCGTACGGGGTGACGCAG-3' (underline; *Kpn*I site, double underline; mutation of RARE), Reverse, 5'-TTTATGTTTTTGGCGTCTTCC-3'. The PCR product was digested by *Kpn*I and *Nco*I, and ligated into pGL3 basic vector digested by the same enzymes. To prepare -1.1 kb/pGL3 with mutated RARE, the following primer sets were used. Upper primer A 5'-CTTCCTGGGGAGGCGGTGAATTGGGGCCAC-3', Lower primer B 5'-TCACCCCGTACGAAATTTGAAATTTCCGCGGAGGGGACGGGC-3', Upper primer C

5'-CGTCCCCTCCGCGGAAATTTCAAATTTCGTACGGGGTGACGCAG-3', Lower primer D 5'-TCCAGCGGTTCCATCTTCCAGCGGATAGAATGGCG-3' (double under line; mutated RARE). Using -1.1 kb/pGL3 as a template, PCR was performed using the two primer sets respectively (A and B, and C and D). Each PCR product was purified, and the mixture of these PCR products was used as the second PCR template with the primer set of A and D. The PCR product was digested by *Blp*I and *Nco*I, and ligated into pGL3/-1.1 kb digested by the same enzymes.

Luciferase assay

SH-SY5Y cells were plated at a density of $5x10^5$cells/ml. After 2 days of incubation, 0.5 μg of promoter construct and 2 μg of *β-galactosidase* expression vector were cotransfected into cells using lipofectin reagent (Invitrogen) according to the manufacturer's protocol. After transfection, supernatant was replaced to Dulbecco's modified Eagle's medium supplemented with 10% FBS with or without ATRA, then cultured for a further 24 h. Luciferase activity and β-galactosidase activity were measured, and the relative luciferase activity was demonstrated as Luc/β-gal.

Electrophoresis mobility shift assay

Electrophoresis mobility shift assay (EMSA) was performed as described previously[23,24]. Nuclear fraction was prepared from control SH-SY5Y and ATRA-treated SH-SY5Y cells, respectively. The biotin-labeled double-strand probe covered the retinoic acid responsive elements (RARE) described in Fig. 5. One μg of nuclear protein and 600 fmol of biotin-labeled probe were incubated at room temperature for 40 min. Cold probe was used for competition experiment (x20 excess). For supershift experiments, anti-COUP-TFI antibody (N-19, sc-6575x, Santa Cruz), anti-RXRα antibody (D-20, sc-553x, Santa Cruz), and anti-RARα antibody (C-20, sc-551x, Santa Cruz) (0.2 μg each/sample) were used.

DNA pull-down assay using biotin-labeled RARE probe

Nuclear extracts (100 μg) from SH-SY5Y cells with or without ATRA treatment for 24 h were mixed with 60 pmol of biotin-labeled double strand probe as described above and 15 μg of Poly dI-dC in DNAP buffer (20 mM HEPES-KOH pH 7.9, 80 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 10% (W/V) glycerol, 0.1% Triton X-100, protease inhibitor) for 60 min on ice. The complexes of probe and binding proteins were collected by adding 50 μl of Dynabeads M-280 streptavidin (Invitrogen Dynal AS, Oslo, Norway) to the mixture, and they were rotating at 4ºC for 30 min. Beads were then washed three times with 500 μl of DNAP buffer. The complex was dissolved in SDS sample buffer and heated at 95ºC for 5 min. Protein components were identified by the Western blotting using anti-COUP-TFI (H-60), anti-RARα (C-20), and anti-RXRα (D-20) antibody.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation assay (ChIP assay) was performed as described previously[23,24]. SH-SY5Y cells with or without ATRA treatment were crosslinked with formaldehyde. For immunoprecipitation, normal IgG, anti-COUP-TFI (N-19), anti-RARα (C–20), anti-RXRα (D–20), and anti-Nuclear receptor corepressor (N-CoR; N-19, sc-1611), anti-Silencing mediator for RARs and TRs (SMRT; N-20, sc-1610) and anti-HDAC3 (N-19, sc-8138) (1 μg each) were added to the lysate and incubated at 4ºC overnight with constant rotation. Immune complexes were extracted, and crosslinking was reversed by heating the elute at 65ºC overnight. The elute was digested with proteinase K at

50ºC for 5 h and extracted with phenol/chloroform/isoamylalchohol. DNA was purified by ethanol precipitation. The *CERK* promoter region was amplified by PCR using the following primer set: Forward, 5'-GTCCCCTCCGCGGTCCCC-3', reverse, 5'-GCTTCACCCACAGCACGGATT-3'.

Cellular C1P measurement

SH-SY5Y cells grown to 80% confluence in 6 cm dish were incubated with or without 10 μM ATRA for 1 h, and then 10 KBq of L-[3-^{14}C]-serine (GE Healthcare, Japan) was added to the culture medium for 23 h. Total lipids were extracted from the harvested cells using $CHCl_3$-MeOH-1% $HClO_4$ (1:1:1, v/v) by our modification of the method of Bligh and Dyer[25], and subjected to a mild alkaline hydrolysis. The alkali-stable lipids were separated by high performance thin-layer chromatography with $CHCl_3$-acetone-MeOH-acetic acid-water (10:4:3:2:1, v/v), and the thin-layer plate was exposed to Imaging Plate (BAS-IP MS 2040, Fuji film, Japan). The radioactive bands were visualized with a bio-imaging analyzer (FUJIX BAS-2500, Fuji, Japan), and the radio-intensity of a band of ^{14}C-ceramide 1-phosphate was measured.

Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed using Student's *t* test or one-way factorial analysis of variance and multiple comparison test (Fishers' method) using Stat view ver5 (SAS Institute Inc., Cary, NC, USA).

Results

CERK as inhibitor of ATRA-induced neuronal differentiation

CERK enzyme activity was decreased during ATRA-induced neuronal differentiation of SH-SY5Y cells (Fig. 1a). ATRA but not retinol reduced CERK protein and mRNA level in a time- and concentration-dependent manner (Fig. 1b, c and d). The effect of overexpression of CERK was examined mainly using a stable subclone 17 (Fig. 2a). CERK overexpression moderately counteracted ATRA-induced inhibition of cell proliferation (Figure 2b) as well as ATRA-induced apoptosis when the cells were serum depleted (Figure 2c). We also examined another subclone 11 with similar results (data not shown). On the contrary, siRNA of *CERK* as shown in Fig 2e and f enhanced the effect of ATRA on cell growth inhibition and apoptosis when serum was depleted. Furthermore, Fig. 3 illustrates the effect of overexpression and inhibition of CERK on neuronal differentiation markers, neurite formation and GAP-43 expression level. CERK overexpression decreased and siRNA of *CERK* enhanced ATRA-induced neurite formation (Fig. 3a, b, d, and e). Similarly, GAP-43 expression was modulated with CERK overexpression or siRNA of *CERK* (Fig. 3c and f), suggesting that the modulation of CERK level affected neuronal differentiation.

Decreased CERK promoter activity in ATRA-treated SH-SY5Y cells

The transcription start point of CERK gene in SH-SY5Y cells was determined by 5'RACE, which was the same as that reported in the on-line database (NCBI; NM_022766). Fig. 4a shows that ATRA considerably reduced *CERK* promoter activity. The reduction of *CERK* promoter activity by ATRA was mainly due to the region between -40 bp and -28 bp from the first exon when evaluated by the ATRA(+)/(-) ratio ((a), right). Computer search revealed the presence of the tandem retinoic acid- responsive element (RARE) in this region with one base space illustrated in Fig. 4b (left), which resembled the finding in another study[26]. The introduction of mutation into these RARE erased ATRA-responsive decrease of the promoter activity (Fig. 4b). The introduction of the same RARE mutation into the long -1100 bp promoter showed the loss of ATRA sensitivity, neglecting the possibility of promoter length problem using various lengths of promoters.

EMSA analysis

ATRA induced cellular COUP-TFI, and increased RARα but slightly decreased RXRα (data not shown). Fig. 5a illustrates EMSA using these RARE (illustrated in Fig. 4b) as the labeled oligoprobe. Among the 3 bands (*a, b* and *c*) observed, bands *a* and *b* were increased by ATRA, and were erased with the cold competitor. Mutated RARE oligo probe did not produce bands *a* and *b*. It has been reported that COUP-TFI inhibited retinoic acid activity by blocking the access of the ligand-inducible receptor complex that was either RAR/RXR heterodimer or RXR homodimer depending on the ligand subtype[27,28]. Supershift assay using anti-RARα, anti-RXRα and anti-COUP-TFI antibody (Fig. 5b) illustrates that bands *a* and *b* produced by ATRA were completely erased by anti-COUP-TFI and moderately by anti-RARα and anti-RXRα antibodies, suggesting that COUP-TFI might be the factor behind ATRA-induced suppression of CERK transcription. DNA pull-down assay further revealed that RARα, RXRα and COUP-TFI were bound increasingly to this RARE-like motif after ATRA treatment (Fig. 5c). We also performed DNA pull-down assay using anti-histone deacetylase (HDAC) antibodies. Among them, HDAC3 was increasingly bound to this RARE by ATRA treatment (data not shown).

Effects of siRNA of RARα, RXRα, and COUP-TFI on CERK mRNA and protein level

The effect of siRNAs of *RARα*, *RXRα* and *COUP-TFI* on *CERK* expression was examined (Fig. 6). Scrambled siRNA showed no alternation of reduced *CERK* mRNA level treated with ATRA as observed in ATRA-treated control SH-SY5Y cells. Among 3 siRNAs, siRNA of *COUP-TFI* but not *RXRα* and *RARα siRNA* recovered ATRA-induced decrease of *CERK* mRNA and protein level (Fig. 6b and c).

ChIP assay

In order to confirm EMSA data described above, ChIP assay was performed. Fig. 7a illustrates that ATRA induced binding of all 3 transcription factors (RARα, RXRα and COUP-TFI) examined in EMSA to these RARE sequences. Moreover, corepressors of gene transcription such as HDAC3, N-CoR and SMRT, were increasingly recruited to these RARE region after ATRA treatment. Interestingly, our preliminary experiments suggested that another ATRA-induced differentiation model, HL60 (a human myeloid leukemia cell line), showed some increase of CERK mRNA with ATRA. Our ChIP assay also showed binding of RARα and RXRα but not COUP-TFI in ATRA-induced HL60 cells (Fig. 7a lower part), suggesting the relative ratio of RAR/RXR and COUP-TFI/RXR or RAR might determine the level of CERK transcription in various cell lines.

Overexpression of three transcription factors and immunoprecipitation (IP) followed by Western blotting

Fig. 7b showed the results of transient overexpression of three transcription factors, *RARα*, *RXRα*, *COUP-TFI* and its combination. *COUP-TF1*, *COUP-TF1/RARα* and *COUP-TF1/RXRα* inhibited *CERK* mRNA significantly, whereas *RARα*, *RXRα* and *RARα/RXRα* did not. We tried to determine which transcription factor (in this case, RARα, RXRα and COUP-TFI) was the real component of transcription modulator complex by immunoprecipitation followed by Western blotting. We could not obtain any positive results probably due to the very low cellular protein levels (data not shown). Therefore, we transfected these three transcription factors to SH-SY5Y cells transiently, and analyzed the complex formation among these 3 proteins. Fig. 7c illustrates that methoprene acid, a specific agonist of RXRα, as well as ATRA (data not shown), increased the heterodimer formation of both RARα/RXRα and COUP-TFI/RXRα. Similarly, ATRA induced the complex formation between COUP-TFI and RARα.

Discussion

Recent analysis revealed that sphingolipids and their metabolizing enzymes are involved in cellular signaling[29]. Among them, S1P and SPHK have received intense attention because of not only biological interest but also its therapeutic potential[30,31]. On the contrary, the analysis of ceramide kinase (CERK), which belongs to the diacylglycerol kinase superfamily, is limited compared with that of SPHK. CERK and its product, C1P have also been suggested to play an important role in cellular events such as cell growth and inflammation[1,4]. Studies in CERK-knockout mouse have also demonstrated that CERK may play a major role in the control of C1P, ceramide and dihydroceramide levels in vivo[32,33]. CERK-knockout mouse showed reduced body weight but revealed less remarkable phenotypic change, suggesting the presence of the minor complementary pathway in vivo in C1P production[34].

CERK inhibition was reportedly detrimental to cell growth, when a novel CERK inhibitor was used[35]. Interestingly, the brain contains high CERK as well as C1P. It was also reported that CERK was involved in the function of cerebellar Purkinje cells using the CERK knockout mouse model[34]. Moreover, ATRA has been implicated in the development of the nervous system and the limbs[36,37,38]. Our results of stable CERK transformants and siRNA of CERK not only confirmed previous results but also revealed CERK as the unique modulator of neuronal cell function in ATRA-induced SH-SY5Y cells (Fig. 2 and 3). In our ATRA-treated SH-SY5Y cells, CERK might be anti-apoptotic and also suppress ATRA-induced neuronal differentiation. Although *CERK* mRNA and protein expression during normal neuronal development have not been reported, future analysis of this issue might be very interesting to elucidate the function of CERK in the central nervous system. Previously, Neuman *et al*.[39]reported their results analyzing the relationship between COUP-TFI and ATRA-induced neuronal differentiation using a teratocarcinoma cell line, in which COUP-TFI was overexpressed.

We recently reported that *SPHK1* mRNA increased in GDNF-induced neuronal differentiation of a human neuronal cell line, TGW cells[20]. In ATRA-treated SH-SY5Y cells, we did not observe any change in the *SPHK1* mRNA level (Fig. 1d), suggesting the possibility that CERK and SPHK1 play non-overlapping roles in neuronal differentiation. Reports of *CERK* mRNA expression during cell differentiation model are few. There is only one brief report of the decrease of *CERK* mRNA and enzyme activity during TPA-induced macrophage-like differentiation of HL60, a human leukemia cell line[17]. We found that ATRA increased *CERK* mRNA of HL60 cells. Therefore, the *CERK* mRNA level modulated with ATRA might be cell context-dependent.

Vitamin A and its major metabolite, ATRA, are essential for embryonic patterning and development[40]. Network formation during early brain development is a complex process. The cellular effect of ATRA is thought to be primarily mediated by two classes of nuclear retinoid receptors, RARs and RXR[41], which act as transcription factors to regulate gene expression. However, the interaction of these retinoid receptors can be modulated by other transcription factors such as COUP-TFs, which are present in the nervous system[42,43].

Our analysis clearly showed the decreased *CERK* mRNA occurred at the transcription level and that tandem RAREs were responsible for this ATRA sensitivity (Fig. 4). Previous study also showed that ATRA modulates the expression of COUP-TFI[44]. In our model, ATRA increased cellular RARα and COUP-TFI, but moderately decreased RXRα protein (data not shown). However, DNA pull-down assay suggested increased binding to these RARE with all three transcription factors. Our siRNA analysis (Fig. 6) showed that COUP-TFI is one of the major factors in ATRA-induced *CERK* mRNA inhibition. COUP-TFI involvement in

modulating ATRA-induced differentiation has been reported in multiple cell types[45,46]. Overexpression of COUP-TFI inhibited ATRA-induced neuronal differentiation of a mouse teratocarcinoma cell line, PCC7[39], which might be apparently opposite to our present results that ATRA-induced COUP-TFI suppressed CERK followed by neuronal differentiation. However, their results were obtained from a stably overexpressed and selected cell line. It is thought that ATRA-induced COUP-TF1 of SH-SY5Y cells was not as high as that of their overexpressed PCC7. In our case, COUP-TFI might play as the fine modulator of ATRA-iuduced differentiation. Moreover, it was reported that the expression level and the timing of expression were important for the ATRA-induced differentiation process of P19 cells[47].

Although a study[28] using in-vitro produced proteins denied the possibility of heterodimer formation of COUP-TFI and RXR or RAR, our supershift assay (Fig. 5b) and IP-western blotting analysis (Fig. 7c) suggested that ATRA increased COUP-TFI/RXRα, COUP-TFI/RARα, and RARα/RXRα complex formations and their binding to these RAREs. Because two RAREs are located in tandem with one bp spacer, the comparison between HL60 and SH-SY5Y cells induced with ATRA (Fig. 7a) suggests that the relative ratio between COUP-TFI/RXRα, COUP-TFI/RARα, and RARα/RXRα could determine the CERK mRNA level. Although we could not rule out the possibility of the triple complex formation, overexpression experiment of *RARα*, *RXRα*, *COUP-TF1,* and their combination (Fig. 7b) supports the results of siRNA experiments shown in Fig.6.

In transcription suppression, the increased binding of corepressors has been reported[48,49]. The large complex formation of COUP-TFI, RXRα and HDAC3, N-CoR and SMRT was strongly suggested, although we could prove this huge complex formation directly. There might be differences of co-regulators or co-suppressors depending on the cell lines used. Data on ATRA-induced HL60 cells (Fig. 7a right lower part) well explain the cell line-dependence of ATRA-induced CERK transcriptional regulation.

It is interesting to know whether C1P/Ceramide ratio is important as the case of S1P/Ceremide balance. However, C1P measurement is still problematic. Very recently, it was reported the difficulty of C1P measurement by HPLC ESI-MS/MS and was also shown that C1P level in previous reports might be overestimated[50]. Therefore, we just tried metabolic labeling experiment, although the same report[50] suggested that C1P measured by metabolic labeling was only a part of total cellular CIP. Our preliminary results showed that ATRA reduced cellular C1P to 60% of control cells. Furthermore, we found that ATRA also induces other sphingolipid metabolic enzymes than CERK (Tanaka *et al*. in preparation). Therefore, it remained to be determined that CERK inhibition by ATRA really reduced total cellular C1P.

Taken together, we demonstrated for the first time that CERK was antagonistic to ATRA-induced neuronal differentiation and that a tandem RARE located in the 5' promoter region was responsible for ATRA responsiveness. Furthermore, we found that increased COUP-TFI induced with ATRA was responsible for the inhibition of CERK gene transcription. Our finding suggests the possible role of CERK in the early physiological process of normal neuronal differentiation as well as the therapeutic implication of ATRA for neuronal malignancies with RA receptors.

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Figure legends

Figure 1. CERK expression during ATRA-induced differentiation of SH-SY5Y cells.

(a) SH-SY5Y cells were cultured with or without 10 μM of ATRA for 12 h. CERK activity was measured according to the Materials and methods. (b) SH-SY5Y cells were cultured with various concentration of ATRA. CERK and β-actin protein level were analyzed by Western blotting. (c) SH-SY5Y cells were cultured with or without 10 μM of ATRA or retinol, respectively for 24 and 48 h. CERK and β-actin protein level were shown. (d) Quantitative RT-PCR was performed according to the Materials and methods to measure *CERK* and *SPHK1* mRNA of SH-SY5Y cells treated with or without 10 μM of ATRA or retinol for 24 and 48 h. Data was shown as the mean from two dependent experiments performed in duplicate. mRNA level of untreated cells were regarded as 1.0.

Figure 2. Effects of modulation of CERK level in ATRA-treated cell proliferation and survival of SH-SY5Y cells

Stable transformants of CERK overexpressed SH-SY5Y cells were established as described in the Materials and methods.

(a) Western blotting of CERK of representative CERK-overexpressed subclone 17 (sc17). (b) Mock-SH-SY5Y and sc17 were cultured with or without 10 μM of ATRA. Cell proliferation was determined on day 4 and day 6 by WST-1 assay as described in the Materials and methods. The initial cell concentration was regarded as 1.0. (c) In serum depleted culture, Mock-SH-SY5Y and sc17 were treated with or without ATRA. On day4 and day6, cell death was determined by LDH cytotoxic test (Wako Pure Chemical Industries., Osaka, Japan). One hundred % of cell death was determined when total cells in the well were killed. Statistical significances were calculated as described in the Materials and methods.

* denotes $p<0.01$, ** shows $p<0.005$. (d) Western blotting of CERK protein treated with scrambled siRNA or siRNA of *CERK*. (e) and (f) Effect of siRNA of *CERK* on cell proliferation or cell death. SH-SY5Y cells were transfected with either scrambled siRNA or siRNA of *CERK* according to the Materials and methods. Cell proliferation and cell death were measured in the similar way as (b) and (c).

Figure 3. Effects of modulation of CERK expression on neurite formation and GAP-43 expression.

In the similar way as Fig. 2, CERK-overexpressed SH-SY5Y and CERK-repressed SH-SY5Y cells with siRNA of CERK were examined for their neurological phenotype when treated with 10 μM of ATRA for 24 h. (a), (b), (d) and (e) Neurite formation was illustrated and the percentage of the neurite positive cells was shown. Photos are representative of three experiments with the similar results. (c) and (f) another neurological phenotype, GAP-43 was analyzed in these cell lines treated with or without ATRA for 24 and 48 h by Western blotting.

Figure 4. Promoter activity of 5' promoter region of hCERK gene of SH-SY5Y cells.

(a) Using -1.7 kb and various truncated promoter region of CERK/Luc, respective promoter activity was examined in SH-SY5Y cells treated with or without 10 μM of ATRA according to the Materials and methods. Cells were cultured in triplicate and experiments were repeated at least three times. The mean +/- SD was shown. The data of relative luciferase activity

(luc/β-gal) of -3 bp Luc of control SH-SY5Y cells was regarded as 1.0. Solid bar denotes ATRA (+), whereas open bar was cells without ATRA treatment. On the right, the ratio of ATRA +/- were illustrated.
(b) Mutated RARE was introduced into -40 bp Luc vector (left side) as described in the Materials and methods. Relative luciferase activity and ATRA +/- ratio were shown on the right. Solid bar was ATRA (+). Open bar shows control cells. Data was derived from three independent experiments performed in triplicates. The data of relative luciferase activity (luc/β-gal) of -3 bp Luc of control SH-SY5Y cells was regarded as 1.0. * denotes the statistical significance, $p< 0.001$. N.S; not significant

Figure 5. Electrophoresis mobility shift assay (EMSA) and DNA pull-down assay.

(a) EMSA was performed according to the Materials and methods. Wild type oligo (oligo wt) and RARE mutated oligo (oligo RARE mut) were illustrated. (b) EMSA using nuclear proteins of control and ATRA-treated SH-SY5Y cells. Three bands observed in ATRA-treated cells were named as *a*, *b* and *c*, respectively. In competition experiments, cold oligo (x20 fold excess) was added. (c) Supershift experiment. anti-RARα (0.2 μg /sample), anti-RXRα (0.2 μg/sample) or anti-COUP-TFI (0.2 μg/sample) antibody was added to each nuclear extract for 20 min at room temperature before mixing labeled oligo. (d) DNA pull-down assay. Nuclear protein of SH-SY5Y cells with or without ATRA treatment for 24 h and biotin labeled oligo probe as described were incubated for 60 min on ice. Dynabeads M-280 streptavidin was added to the mixture, and DNA-protein complex was collected on a magnetic stand and washed three times with DNAP buffer. Proteins complexed with labeled DNA were analyzed by Western blotting using anti-RARα, anti-RXRα or anti-COUP-TFI antibody, respectively.

Figure 6. Effects of siRNA of *RARα, RXRα*, and *COUP-TFI* on *CERK* mRNA and protein.

Scrambled siRNA or siRNA of *RARα*, *RXRα* and *COUP-TFI* were transfected and treated with ATRA for 24 h.
(a) Effects of siRNA were confirmed by Western blotting. (b) Quantitative RT-PCR of *COUP-TFI* mRNA was performed according to the Materials and methods. The mean +/- SD was calculated, and mRNA level of untreated cells transfected with scrambled siRNA or mock-expression vector was regarded as 1.0.

Figure 7. Chromatin immunoprecipitation (ChIP) assay, overexpression of three transcription factors and immunoprecipitation (IP) followed by Western blotting.

(a) ChIP assay was performed using SH-SY5Y cells with or without ATRA treatment for 24h. Normal IgG, anti-RARα, anti-RXRα, anti-COUP-TFI, anti-HDAC3, anti-N-CoR, or anti-SMRT antibody was used for immunoprecipitation. The expected PCR product was 135 bp covering RARE (upper part). ChIP assay was also performed using HL60 cells with ATRA treatment for 24 h (lower part).
(b) SH-SY5Y cells were transfected transiently with mock-, *RARα*-, *RXRα*-, *COUP-TF1* expression vector, respectively or in combination as shown in the figure. After 24 h treatment with ATRA, CERK mRNA was measured by the quantitative RT-PCR method. The mean +/- SD was shown. *CERK* mRNA of mock-transfected cells treated with ATRA was regarded as 1.0.

(c) IP followed by Western blotting. Complex formation of cellular proteins was examined with immunoprecipitation of cellular proteins followed by Western blotting. SH-SY5Y cells were co-transfected with *COUP-TFI*, *RARα*, and *RXRα* expression vectors, and treated with either methoprene acid or ATRA for 24 h. Methoprene acid was the specific ligand of RXRα. IP was carried out using anti-RXRα antibody or anti-COUP-TFI antibody. Precipitated proteins were visualized by the Western blotting.

Figure 1

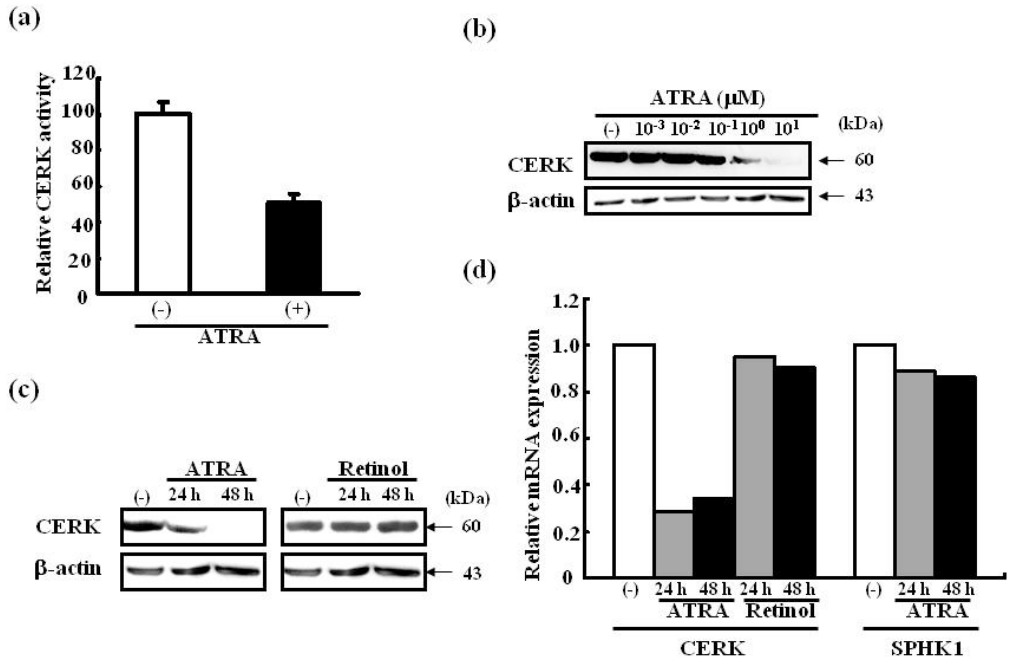


Figure 2

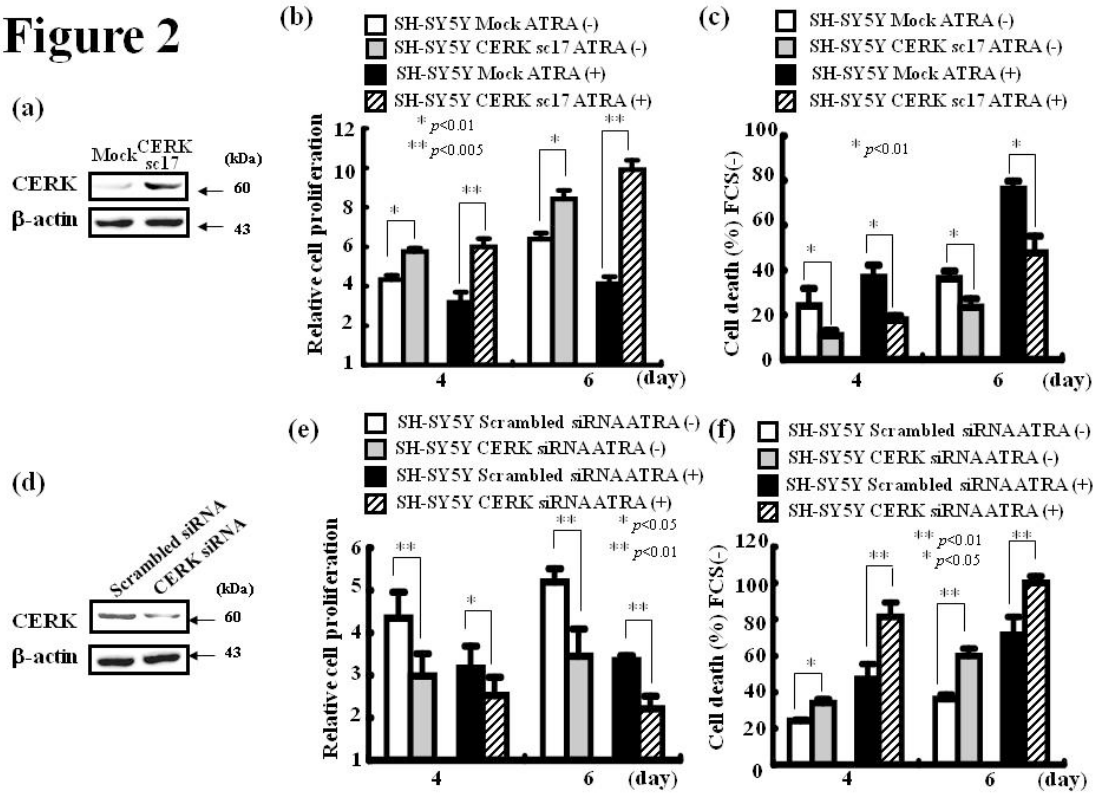


Figure 3

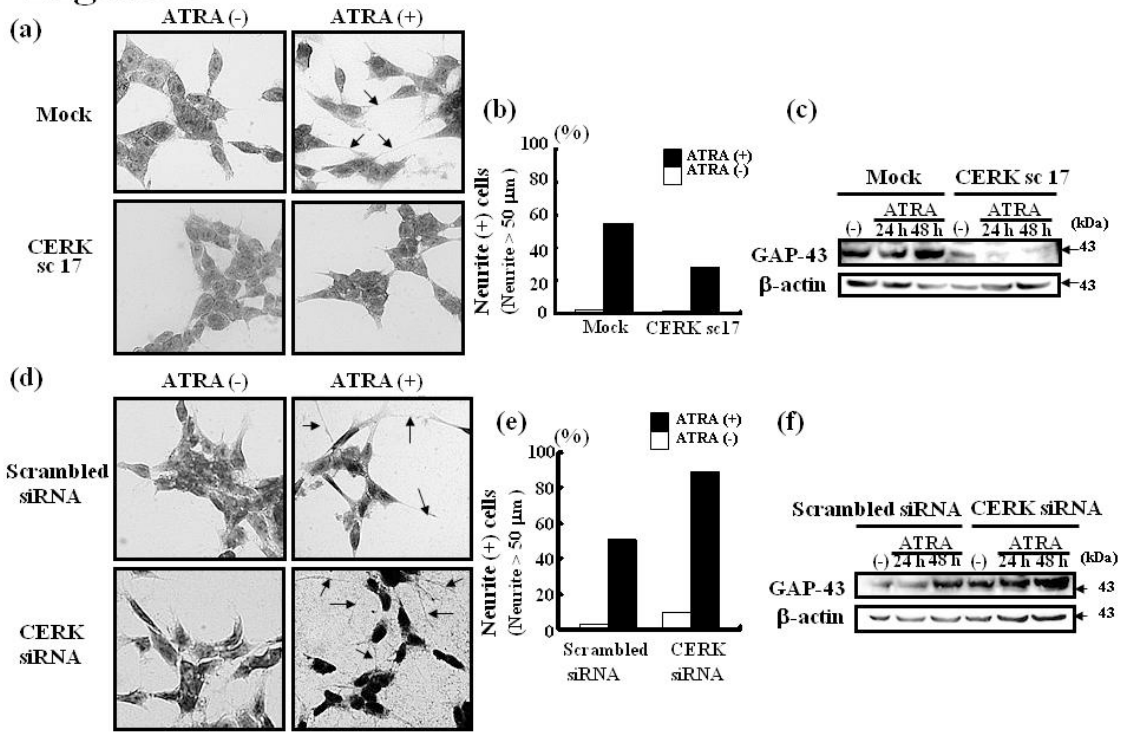


Figure 4

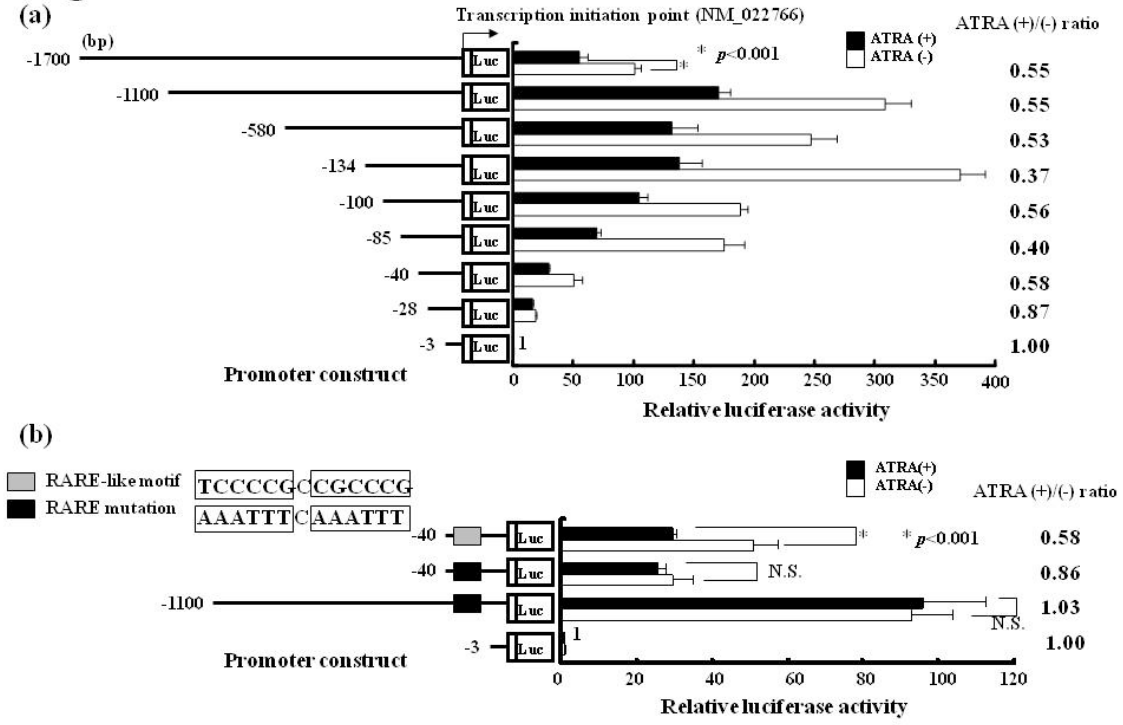


Figure 5

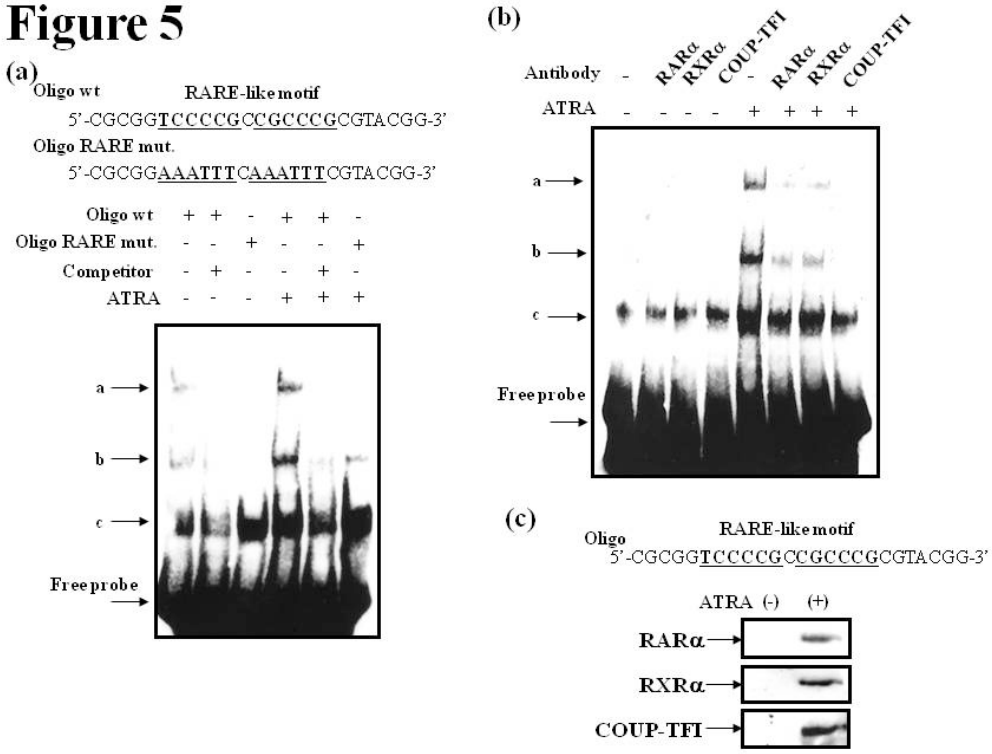


Figure 6

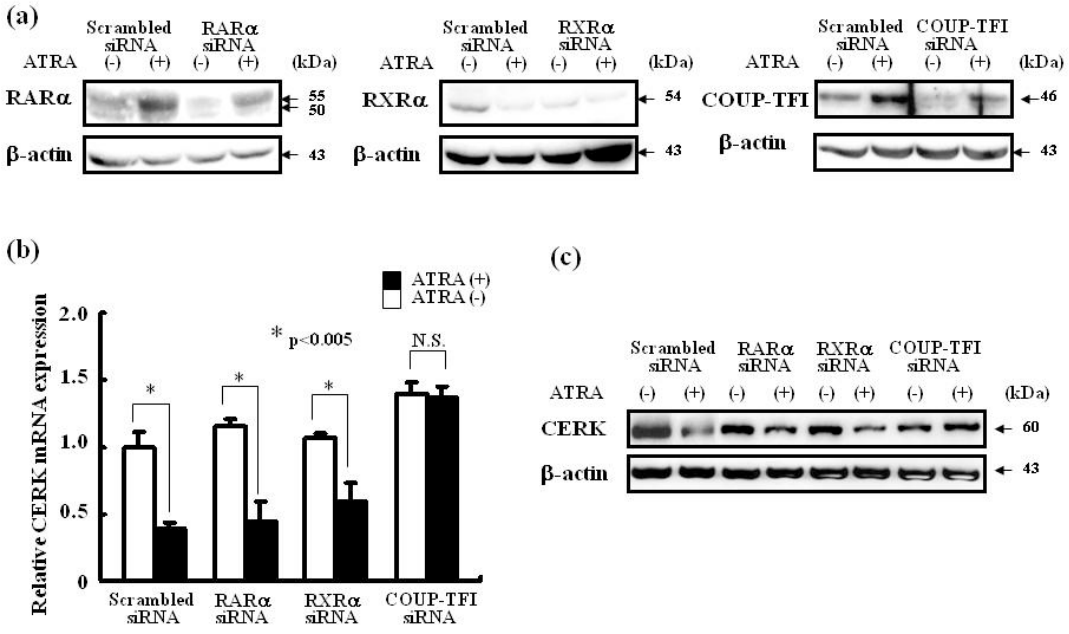
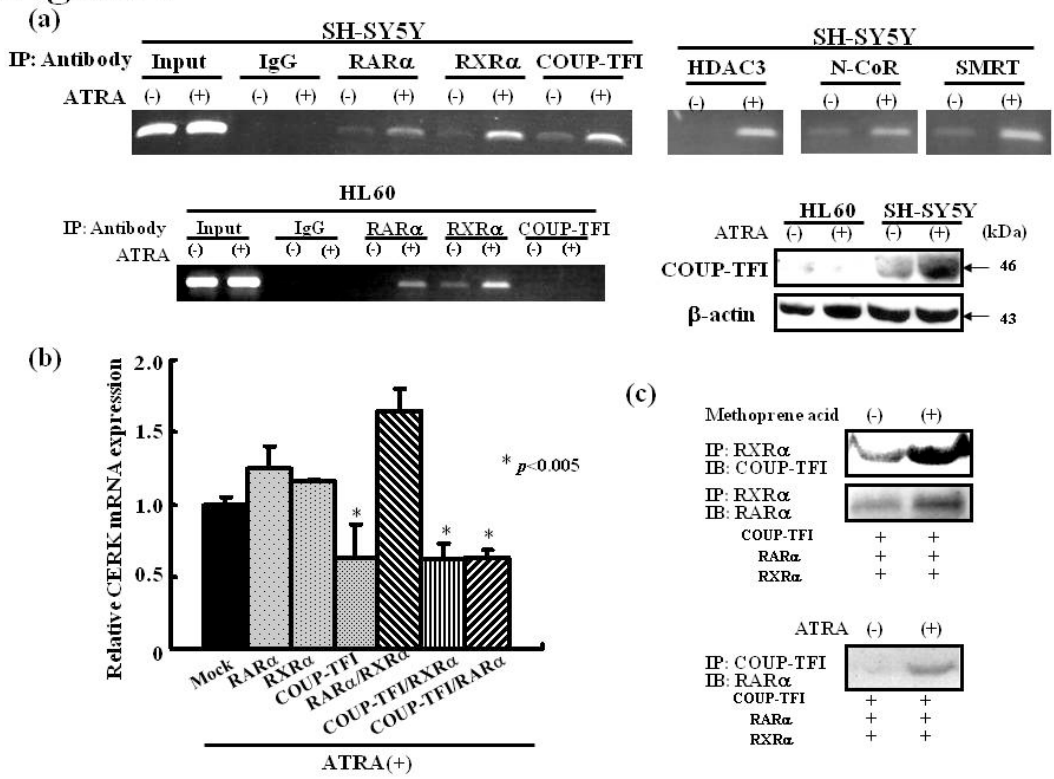


Figure 7


(a)
SH-SY5Y
IP: Antibody
Input
IgG
RARα
RXRα
COUP-TFI
ATRA
(-) (+)
SH-SY5Y
HDAC3
N-CoR
SMRT
HL60
HL60
SH-SY5Y
(kDa)
COUP-TFI
46
β-actin
43
(b)
Relative CERK mRNA expression
2.0
1.5
1.0
0.5
0
* p<0.005
Mock
RARα
RXRα
COUP-TFI
RARα/RXRα
COUP-TFI/RXRα
COUP-TFI/RARα
ATRA(+)
(c)
Methoprene acid
IP: RXRα
IB: COUP-TFI
IP: RXRα
IB: RARα
COUP-TFI
RARα
RXRα
IP: COUP-TFI
IB: RARα