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Transcriptional regulation of neutral sphingomyelinase 2 in all-*trans* retinoic acid-treated human breast cancer cell line, MCF-7

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Hiromi Ito[1], Kouji Tanaka[2], Kazumi Hagiwara[3], Misa Kobayashi[3], Asuka Hoshikawa[3], Naoki Mizutani[3], Akira Takagi[1], Tetsuhito Kojima[1], Sayaka Sobue[4], Masatoshi Ichihara[4], Motoshi Suzuki[5], Keiko Tamiya-Koizumi[1], Mitsuhiro Nakamura[6], Yoshiko Banno[7], Yoshinori Nozawa[8] and Takashi Murate[1,*]

[1]Department of Medical Technology, Nagoya University School of Health Sciences; [2]Department of Oncology, Nagoya City University Graduate School of Pharmaceutical Sciences; [3]Department of Pathophysiological Laboratory Sciences, Nagoya University Graduate School of Medicine, Nagoya; [4]Chubu University College of Life and Health Sciences, Kasugai; [5]Division of Molecular Carcinogenesis, Nagoya University Graduate School of Medicine, Nagoya; [6]Laboratory of Drug Information, Gifu Pharmaceutical University; [7]Department of Dermatology, Gifu University Graduate School of Medicine, Gifu; and [8]Gifu International Institute of Biotechnology and Tokai Gakuen University, Kakamigahara, Japan

*Takashi Murate, Nagoya University School of Health Sciences, Daiko-minami 1-1-20, Higashi-ku, Nagoya 461-8673, Japan. Tel/Fax: +81-52-719-1186, email: murate@met.nagoya-u.ac.jp

Effects of all-*trans* retinoic acid (ATRA) on sphingomyelinase expression were examined using MCF-7 (ATRA-sensitive) and MDA-MB-231 (ATRA-resistant) breast cancer cells. Increased NSMase activity, NSMase2 mRNA and protein were observed in ATRA-treated MCF-7 but not in ATRA-treated MDA-MB-231. Increased NSMase2 mRNA of ATRA-treated MCF-7 was mostly due to enhanced transcription. Promoter analysis revealed the important 5′-promoter region of NSMase2 between −148 and −42 bp containing three Sp1 sites but no retinoic acid responsive elements. Experiments using mutated Sp1 sites of the NSMase2 promoter, Mithramycin A (a Sp inhibitor) and Sp family over-expression demonstrated the importance of Sp family protein and the three Sp1 sites for ATRA-induced NSMase2 transcription of MCF-7 cells. Although no quantitative change of bound Sp1 on NSMase2 promoter region after ATRA treatment was detected, Sp1 phosphorylation (activation) by ATRA was observed. Interestingly, PKCδ was involved in ATRA-induced increased NSMase2 transcription. ATRA-induced PKCδ phosphorylation and then activated PKCδ phosphorylated Sp1. Chromatin immunoprecipitation (ChIP) assay showed Sp1, RARα and RXRα complex formation in MCF-7 cells regardless of ATRA treatment and ATRA-induced acetylated histone H3 of the 5′-promoter. Thus, NSMase2 mRNA expression enhanced by ATRA was due to increased transcription via phosphorylated Sp1 caused by PKCδ activation, followed by chromatin remodelling with histone H3 acetylation.

Keywords: all-*trans* retinoic acid/chromatin remodelling/MCF-7 cell/neutral sphingomyelinase 2/promoter analysis.

Abbreviations: ASMase, acid sphingomyelinase; ATRA, all-*trans* retinoic acid; ER, oestrogen receptor; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; NSMase2, -neutral sphingomyelinase 2; RAR, retinoic acid receptor; RARE, retinoic acid-responsive element; RXR, retinoid X receptor.

Hormone-dependent growth has been observed in some human cancers including prostate cancer and breast cancer (*1*). The former is androgen sensitive, whereas the latter oestrogen-dependent. Therefore, therapy with hormone antagonists is selected for the cases with these hormone-sensitive cancers. Oestrogen receptors (ERs) and progesterone receptors (PRs) are frequently present in breast cancers and function as prognostic indications for response to treatment (*2*). It has been reported that 50–60% of the women with ER-positive breast cancers benefit from endocrine therapy (*3*). However, hormone insensitivity was frequently experienced during the disease progression and continuous treatment. Therefore, it is of clinical importance to overcome the therapeutic limitation of these hormone-dependent cancer cells.

Retinoids, such as all-*trans* retinoic acid (ATRA), are derivatives of vitamin A that induce differentiation of acute promyelocytic leukaemia (APL) cells and inhibit growth of other cancer cells including breast cancer cells (*4–7*). The anti-cancer effects of retinoids have been intensely investigated. Apart from leukaemia and breast cancer, the effectiveness of retinoid therapy has been reported in prostate cancer, skin cancer and lung cancer (*8–10*). Furthermore, retinoids may also be beneficial for cancer prevention (*11, 12*).

ERα, RARα and cellular retinoic acid-binding protein are reportedly co-ordinately regulated (*13*). ERα-positive breast cancer cells have been found to increase their sensitivity to ATRA-induced growth inhibition (*14*). It is also of note that the RARα expression level was lower in breast cancer cells resistant to

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ATRA as compared with their sensitive counterpart (*15–17*). However, the signalling between RARα and ERα and also the mechanism of ATRA-induced cell growth inhibition remain to be elucidated. Effects of retinoids are mostly mediated by their binding to the nuclear receptors RAR (retinoic acid receptor) and RXR (retinoid X receptor), which form the heterodimer that activates transcription of genes containing retinoic acid response elements (RAREs) in their promoter regions (*18*), although some other mechanisms not connected with RAREs have been reported (*19–21*).

Ceramide has been recognized as a potent proapoptotic mediator in eukaryotic cells (*22*, *23*). There are three metabolic pathways to produce ceramide; the sphingomyelin degradation pathway, *de novo* synthesis and the salvage pathway. The relationship between cellular ceramide and ATRA-induced signal transduction has been reported in several model systems (*24–26*). In haematological diseases, decrease of neutral sphingomyelinase 2 (NSMase2), which hydrolyses sphingomyelin to produce ceramide, was reported in myelodysplastic syndrome that involves a pre-leukaemic state and *de novo* AML patient (*27*). However, the expression levels of respective SMases of other clinical samples have not been reported. Considering that ATRA could suppress proliferation of leukaemic cells and cancer cell lines, the elucidation of the SMase expression mechanism is of interest and also clinically important.

The role of ceramide in ATRA-induced cancer cell growth arrest has recently been reported using a human breast cancer cell line, MCF-7 and emphasized the suppression of S6 kinase by enhanced NSMase2 expression (*28*). However, the difference of sphingolipid metabolism between ATRA sensitive and ATRA-resistant breast cancer cells remains to be fully elucidated. This issue is also clinically important to elucidate the mechanism of transition of breast cancer cells from ATRA sensitive to ATRA resistant. In the current study, we examined the effects of ATRA on sphingomyelinase expression and ceramide level of two breast cancer cell lines, MCF-7 (ATRA sensitive) and MDA-MB-231 (ATRA-resistant). It was shown that ATRA was effective only in MCF-7 cells and that neutral sphingomyelinase 2 (NSMase2) mRNA but not acid sphingomyelinase (ASMase) mRNA increased in ATRA-treated MCF-7 cells. We focused on elucidation of the transcriptional control mechanism. Our current analysis revealed that ATRA induced a unique mechanism for the NSMase2 transcription and the critical involvement of Sp family transcription factor. It was also shown that PKCδ activated by ATRA–phosphorylated Sp1, followed by the chromatin structure remodelling, finally lead to the increased NSMase2 transcription. Considering our previous report showing that ATRA-induced ASMase transcription of a leukaemia cell line, NB4 (*26*), the regulatory mechanism of SMase transcription by ATRA is heterogeneous and cell type dependent. Here, we have discussed this unique mechanism underlying the NSMase2 transcriptional regulation in ATRA-treated MCF-7 cells.

Materials and Methods

Reagents

ATRA and Mithramycin A were purchased from Sigma-Aldrich (St Louis, MO, USA). TTNPB and metoprane acid were from Enzo Life Sciences Inc. (Farmingdale, NY, USA). U0126 was from Promega (Madison, WI, USA). Calphostin C, SB203580, SP600125, Gö6976 and Rottlerin were from Calbiochem (La Jolla, CA, USA). Anti-NSMase2, anti-Sp1, anti-Sp3, anti-RARα, anti-RXRα, anti-ERα, anti-PKCα, anti-PKCβI and anti-PKCδ antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-phospho-PKCα/β antibody was from New England Biolabs Inc. (Beverly, MA, USA) Anti-phosphoserine polyclonal antibody (HRP-conjugated) was from Assay Designs (Enzo Life Sciences). Anti-phosphorylated-PKCδ antibody, anti-rabbit IgG HRP-linked antibody and anti-mouse IgG HRP-linked antibody were from Cell Signaling Technology (Danvers, MA, USA). Anti-β-actin antibody was from BioVision (Mountain View, CA, USA).

Cell lines

The culture condition of a human breast cancer cell line, MCF-7, was described previously (*29*). ATRA-resistant human breast cancer cell line, MDA-MB-231, derived from ATCC was cultured in 5% FCS in DMEM (Wako Pure Chemical Industries, Ltd. Osaka, Japan).

Cellular viability

MCF-7 and MDA-MB-231 cells were plated in triplicate in 24 well (2.0×10^5/ml) and cultured for 24 h before ATRA was added. The viable cell number was counted using trypan blue dye exclusion test.

siRNA and transfection

siRNA of RARα was purchased from Sigma Genosys (Hokkaido, Japan). The sequence was the following: RARα sense 5′-GAACATGGTGTACACGTGT-3′, anti-sense 5′-ACACGTGTACACCATGTTC-3′. Fifty picomoles siRNA was transfected using Lipofectamine^{TM} RNAiMAX (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer. Control siGENOME Non-Targeting siRNA#1 and 3 were used as control (Thermo Fisher Scientific Inc., Walham, MA, USA).

Western blotting

Western blotting was performed as described before (*29*). Protein samples were dissolved in lysis buffer and separated on 10% or 15% SDS–PAGE and transferred to PVDF membrane. Membranes were blocked 1–5% dry milk in phosphate-buffered saline (PBS) with 0.05% Tween-20 at room temperature for a few hours. Primary antibodies used were as follows: anti-NSMase2 (1:2000), anti-Sp1 (1:1000), anti-Sp3 (1:1000), anti-RARα (1:1000), anti-RXRα (1:1000), anti-ERα (1:1000), anti-PKCα (1:1000), anti-PKCβI (1:1000), anti-PKCδ (1:1000), Anti-phospho-PKCα/β (1:1000), Anti-phospho-PKCδ (1:1000), anti-phosphoserine (1:1000) and anti-β-actin (1:2000) antibodies. Secondary antibodies were anti-rabbit IgG

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HRP-linked antibody or anti-mouse IgG HRP-linked antibody (1:1000–2000). Proteins were detected by Immobilon Western Chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA, USA).

Quantitative RT–PCR of SMase mRNA

Quantitative RT–PCR and semi-quantitative RT–PCR of human NSMase2 and ASMase were performed as reported before (*27*, *29*). The primer sequences were as follows: NSMase2 forward 5′-ACT TTGATAACTGCTCCTCTGAC-3′, reverse 5′-TTCG TGTCCAGCAGAGTACC-3′; ASMase forward 5′-A CTTTGATAACTGCTCCTCTGAC-3′, reverse 5′-TT CGTGTCCAGCAGAGTACC-3′; GAPDH forward 5′-CAGGAGCGAGATCCCTCCAA-3′ and reverse 5′-CCCCCTGCAAATGAGCCC-3′.

Cellular ceramide measurement

The method of liquid chromatography followed by tandem mass spectrometry (LC–MS/MS) for analysis of cellular ceramides was reported before (*29*).

SMase enzyme activity

ASMase and NSMase enzyme activities were measured according to the method described previously (*26*).

Nuclear run-on assay

Nuclear run-on assay was performed as described previously (30). MCF-7 cells were treated with 10 μM ATRA for 6 h. Harvested cells were re-suspended in 4 ml of Nonidet P-40 (NP-40) lysis buffer [10 mM Tris–HCl (pH 7.4], 10 mM NaCl, 3 mM $MgCl_2$ and 0.5% NP-40, 150 mM Sucrose] for 10 min at 4°C. After washing with NP40(−) lysis buffer, the pellet was re-suspended in 100 μl of freezing buffer [50 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, 40% glycerol]. Next, 20 μl of transcription buffer [20 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 4 mM DTT, 200 mM KCl, 200 mM Sucrose, 20% glycerol] and 4 μl of 10× biotin RNA labelling mix containing biotin-16-UTP (Roche, Basel, Switzerland) were added. They were incubated at 29°C for 30 min and then Total RNA was isolated. The RNA dissolved in 50 μl of RNase-free water was reacted with Dynabeads M-280 Streptavidin (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA) resuspended in 50 μl of binding buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA, 2 M NaCl] at room temperature for 2 h. Beads were washed twice using 2× SSC containing 15% formamide for 15 min and once using 2× SSC for 5 min. After the beads were dissolved in RNase-free water, quantitative RT–PCR was carried out.

NSMase2 mRNA half-life

NSMase2 mRNA half-life was measured according to the method described previously (*30*). MCF-7 cells were treated with or without 10 μM ATRA for 24 h before 5 μg/ml actinomycin D treatment. After an indicated incubation period (1 and 6 h), total RNA was isolated for quantitative RT–PCR.

NSMase2 promoter cloning and promoter analysis

The 5′-promoter region between −984 bp and +109 bp of NSMase2 was analysed in the current study, and the preparation of wild-type NSMase2 5′-promoter as well as truncated or mutated forms was described before (*29*). DNA transfection was performed by the calcium phosphate precipitation method. Five micrograms of various lengths of luciferase vector and 2 μg of β-galactosidase expression vector were co-transfected to MCF-7 cells. Cells were treated with 10% glycerol in DMEM for 1 min 6 h after DNA transfection. Then, cells were treated with or without 10 μM of ATRA for 24 h. The relative promoter activity was expressed as luciferase/β-galactosidase activity.

GAL4 Sp1/3 vectors and analysis of their transcriptional activity

Plasmids, pM-Sp1 and pM-Sp3 were the generous gift of Prof. T. Sakai (Kyoto Prefectural University of Medicine, Kyoto, Japan), and they express the GAL4-binding domain fused to either Sp1 or Sp3 (*31*). Pico molar expresses only the GAL4-binding domain used as a negative control. pG5-luc is a luciferase reporter, which carries five GAL4 DNA-binding sites upstream of E1B minimal promoter and the TATA box. Six hours after DNA transfection (2.5 μg of respective pico molar series vector, 0.5 μg of pG5-luc and β-gal expression vector) by Lipofectamine 2000 (Invitrogen), ATRA was added to the culture medium for another 24 h. Then, the luciferase activity was measured and the relative promoter activity stimulated with either Sp1 or Sp3 was calculated with the internal control of β-gal activity. The relative activity of control pM vector was defined as 1.0.

Electrophoresis mobility shift assay

Electrophoresis mobility shift assay (EMSA) was performed according to the method previously described (*32*). Biotinylated wild-type and mutated oligo probe *a* were previously described (*29*) and shown in Supplementary Fig. S3. Supershift assay was conducted using 2 μg of anti-Sp1, anti-Sp3, anti-RARα and anti-RXRα antibody/sample, respectively. In some experiments, nuclear extract was treated by calf intestinal phosphatase (CIP) (1 U/μg protein, Promega) for 37°C for 30 min to reduce protein phosphorylation.

Phosphorylated Sp1 detection

Whole-cell lysate was prepared with RIPA buffer [150 mM NaCl, 1%NP-40, 0.05% deoxicholic acid, 0.1% SDS, 50 mM Tris (pH 8.0)]. Dynabeads® Protein A (Invitrogen) was added to anti-Sp1 antibody (1 μg) diluted in 200 μl PBS(−) with 0.05% Tween-20 and rotated for 10 min at room temperature. After washing, the sample (500 μg/500 μl RIPA) was added to the beads–antibody complex and was rotated for 10 min at room temperature. After three washes using PBS (−), the sample was eluted to 20 μl of sample buffer and heated for 5 min at 95°C. Western blotting was performed using anti-phosphoserine antibody (1:1000) as described above.

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Preparation of GST-Sp1 and pull down of PKCδ

GST-Sp1 protein was obtained as described (*29*, *33*). Briefly, MCF-7 cells treated with or without ATRA were lysed in NETN buffer [20 mM Tris–HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA and 0.5% NP-40]. Partially purified GST-Sp1 and glutathione particles were mixed with cell lysate overnight at 4°C. Then, the complex with glutathione particles were collected and washed four times by NETN buffer, then lysed in sample buffer as samples of western blotting using anti-PKCδ antibody and anti-phosphorylated PKCδ antibody.

Chromatin immunoprecipitation assay

For the immunoprecipitation, anti-Sp1, anti-Sp3, anti-acetylated histone H3, anti-acetylated histone H4, anti-RARα or anti-RXRα antibody (Santa Cruz, 1 μg/sample) were added to Dynabeads® M-280 Sheep anti-Rabbit IgG (Invitrogen, Carlsbad, CA, USA) and rotated for overnight at 4°C (*29*). Normal rabbit IgG was used as a control IgG. Antibody and beads complex were mixed with samples and rotated for 6 h at 4°C. After DNA extraction, the promoter region containing Sp1 motif was amplified by PCR using the following primers, forward 5′-GGTCTCTG GGGATGTGGTCTT-3′; and reverse 5′-GGCTCTC GCGGCTCTCGGGT-3′.

Statistical analysis

Statistical significances were analysed using Student's *t*-test or one-way factorial analysis of variance and multiple comparison test (Fisher's method) using Excel software (Microsoft).

Results

ATRA induced increase of NSMase2 expression in MCF-7 cells but not in MDA-MB-231 cells

Figure 1 demonstrates the effect of ATRA on cell proliferation, NSMase enzyme activity and ceramide level of MCF-7 and MDA-MB-231 cells. In agreement with the recent report (*28*), ATRA-treated MCF-7 but not MDA-MB-231 cells exhibited growth arrest (Fig. 1A). A significant increase of C24:1 ceramide was observed in ATRA-treated MCF-7 but not in MDA-MB-231, although a mild increase of C18:0 ceramide was observed in ATRA-treated MDA-MB-231 cells (Fig. 1C). These changes paralleled NSMase enzyme activity but not ASMase enzyme activity (Fig. 1B and Supplementary Fig. S1). NSMase2 protein and NSMase2 mRNA were increased by ATRA in MCF-7 cells but not in MDA-MB-231 cells (Fig. 2A and B). ASMase mRNA showed no significant change with ATRA treatment of both cells under our experimental conditions (Supplementary Fig. S1).

Nuclear run-on assay and mRNA half-life measurement

The nuclear run-on assay shown in Fig. 2C demonstrated that the increased NSMase2 mRNA by ATRA is mostly due to the transcription. We also examined NSMase2 mRNA half-life (Supplementary Fig. S2), suggesting that mRNA half-life elongation is the minor contribution to the NSMase2 mRNA levels between the cells treated with or without ATRA.

RARα, RXRα and ERα of MCF-7 and MDA-MB-231 cells

We characterized expression of oestrogen and retinoid receptors of MCF-7 and MDA-MB-231 cells (Fig. 3A). ERα, RARα and RXRα were observed clearly in MCF-7 but very little in MDA-MB-231 cells. ATRA slightly decreased RARα and RXRα expression of MCF-7. TTNPB, a specific RAR agonist and methoprene acid, a specific RXR agonist, were tested instead of ATRA. TTNPB significantly increased NSMase2 mRNA whereas methoprene acid did not, suggesting the major contribution of RARα in ATRA-induced NSMase2 transcription (Fig. 3B). Furthermore, ATRA-induced increase of NSMase2 mRNA was reduced with siRNA of RARα, but not with scrambled siRNA (Fig. 3C). These results indicate that ATRA-induced NSMase2 transcription depends on RARα.

Promoter analysis of NSMase2 gene induced by ATRA

The mechanism of increased NSMase2 expression in MCF-7 treated with ATRA was examined. It was shown that the endogenous and ATRA-induced promoter activity was located mostly between −148 bp and the first exon. Promoter activity of longer reporter construct up to 1-kb upstream did not differ from that of −148 bp construct (Fig. 4A and data not shown). The on-line search did not detect any possible retinoic acid responsive elements (RAREs) but revealed three Sp1 sites in this region. The introduction of a mutation to each Sp1-binding site reduced ATRA sensitivity, suggesting that all three Sp1 sites are important for the endogenous as well as ATRA-induced promoter activity. Since the enhancement of the promoter activity by ATRA was less distinct than that of NSMase2 mRNA (Fig. 2B), the contribution of other sites or factors involving up-regulation of NSMase2 expression was suggested. However, another possibility is that the increased transcription was due to some mechanism by which luciferase reporter vector could not detect efficiently. This issue will be further discussed in the 'Discussion' section.

In MCF-7 cells, Sp1 and Sp3 endogenously bind with this 5′-promoter region (*29*). The Sp family-specific inhibitor, Mithramycin A, abolished ATRA-induced NSMase2 mRNA elevation (Fig. 4B), although ATRA did not affect the Sp1 and Sp3 protein levels (Fig. 4C). In our previous study, Mithramycin A was also shown to reduce the NSMase2 mRNA level of control MCF-7 cells by ~40% (*29*). The transcription promoting activity of Sp1 rather than Sp3 of ATRA-treated MCF-7 cells became more distinct by using GAL4/Sp fusion protein expressing vector (Fig. 4D). Therefore, the Sp family, especially, Sp1, is considered to be responsible for ATRA-induced NSMase2 transcription.

EMSA analysis

We performed EMSA analysis of MCF-7 cells with or without ATRA treatment as reported previously (*29*).

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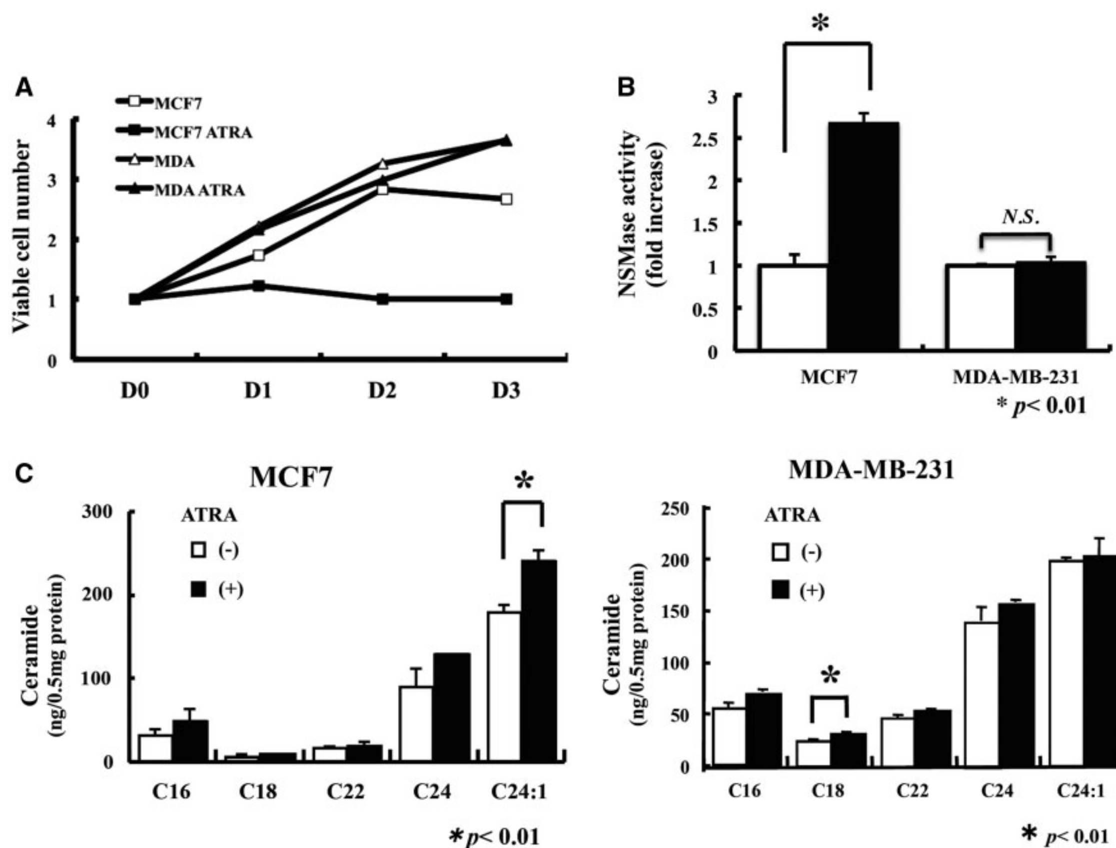

Fig. 1 ATRA-induced growth arrest and NSMase activation of MCF-7 cells. (A) MCF-7 and MDA-MB-231 cells were plated in triplicate in 24-well culture plate 24 h before addition of 10 μM of ATRA. Viable cell number was counted in triplicate with the trypan blue dye exclusion method after detaching cells with PBS (−) containing trypsin–EDTA. Experiments were performed at least three times with similar results. (B) NSMase enzyme activity was measured according to the 'Materials and Methods' section. ATRA (+) (solid column): 10 μM ATRA treatment for 24 h. ATRA (−) (open column): untreated control cells. Activity of control cells was regarded as 1.0. (C) Cellular ceramide levels (shown as ng/0.5 mg protein) were measured using LC/MS–MS according to the 'Materials and Methods' section. MCF-7 and MDA-MB-231 cells were cultured with or without ATRA for 24 h as described above. Cells were collected and samples were prepared. C16, 18, 22, 24 and 24:1 denote respective ceramide species with different carbon chain lengths. An asterisk means statistically significant difference ($P < 0.01$).

The effects of ATRA were examined using Sp1-binding site a (Supplementary Fig. S3a) as the probe. No apparent difference was observed between control- and ATRA-treated MCF-7 cells, and the binding of Sp family proteins, Sp1 and Sp3, was confirmed by supershift experiments using anti-Sp1 antibody and anti-Sp3 antibody (Supplementary Fig. S3b and S3c). Thus, it was concluded that the total amount of Sp1 protein bound with this Sp1-binding site was not changed by the ATRA treatment.

Effects of PKC on NSMase2 mRNA level

To find another mechanism to explain increased NSMase2 mRNA transcription by ATRA, the signalling pathways leading to ATRA-induced NSMase2 transcription were examined using various inhibitors. Pan-PKC inhibitor (Calphostin C) markedly inhibited ATRA-induced NSMase2 mRNA expression, whereas p38 MAPK inhibitor, (SB203580) and MEK inhibitor, (U0127), did not (Fig. 5A). JNK inhibitor (SP600125) showed a rather small increase. Furthermore, PKCδ inhibitor, Rottlerin, but not the PKCα inhibitor, Gö6976, prevented the induction of NSMase2 mRNA by ATRA (Fig. 5B). We have examined phosphorylation of several PKCs by ATRA treatment, and interestingly, phosphorylation of PKCδ in MCF-7 cells was found to be elevated (Fig. 5C) before the up-regulation of NSMase2 (Fig. 5D). ATRA-induced PKCδ phosphorylation (activation) has also been reported previously in NB4, HL60 and MCF-7 cells (*16*). Interestingly, silencing RARα by its siRNA greatly inhibited PKCδ phosphorylation (Fig. 6A).

PKCδ-induced Sp1 phosphorylation

Increased NSMase2 mRNA started after ~6 h of ATRA treatment (Fig. 5D). It is of note that Sp1 phosphorylation by ATRA was started earlier than the increase of NSMase2 mRNA (Fig. 6B). Temporal change of phosphorylated Sp1 (Fig. 6B) paralleled that of PKCδ phosphorylation (Fig. 5C). Sp1 phosphorylation was examined by treating nuclear extract for EMSA analysis with or without CIP (Supplementary Fig. S3d), showing that a significant amount of Sp1 bound to this promoter region was phosphorylated. It also suggests that ATRA-induced phsphorylated Sp1 did not displace the major portion of non-phophorylated Sp1 endogenously bound to this promoter region. PKCδ activation by ATRA-induced higher binding activity with purified Sp1 prepared as

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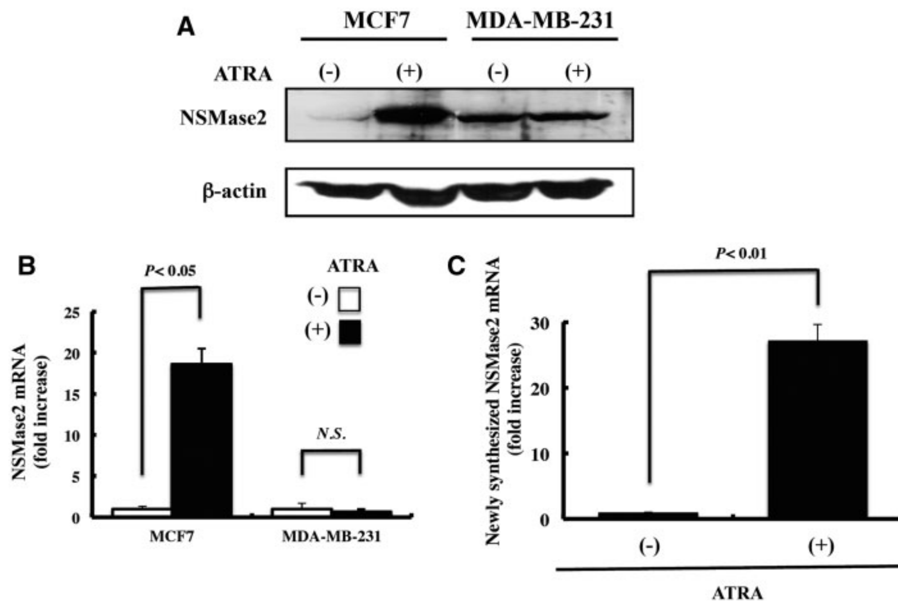

Fig. 2 ATRA-induced NSMase2 gene and protein expression. (A) MCF-7 and MDA-MB-231 cells were treated with or without 10 μM of ATRA for 24 h. Western blotting was performed using anti-NSMase2 antibody. β-Actin was the internal control. (B) Quantitative RT–PCR was performed to measure NSMase2 mRNA levels. The relative SMase mRNAs was calculated as the ratio of SMase mRNA/GAPDH mRNA. Each control sample was regarded as 1.0. Mean ± SD was calculated from three different samples. Experiments were performed three times and statistical significance was analysed. (C) Nuclear run-on assay was performed according to the 'Materials and Methods' section. The results denote quantitative RT–PCR (the mean ± SD) from three different samples. The level of control cells was regarded as 1.0. Statistical significance was analysed.

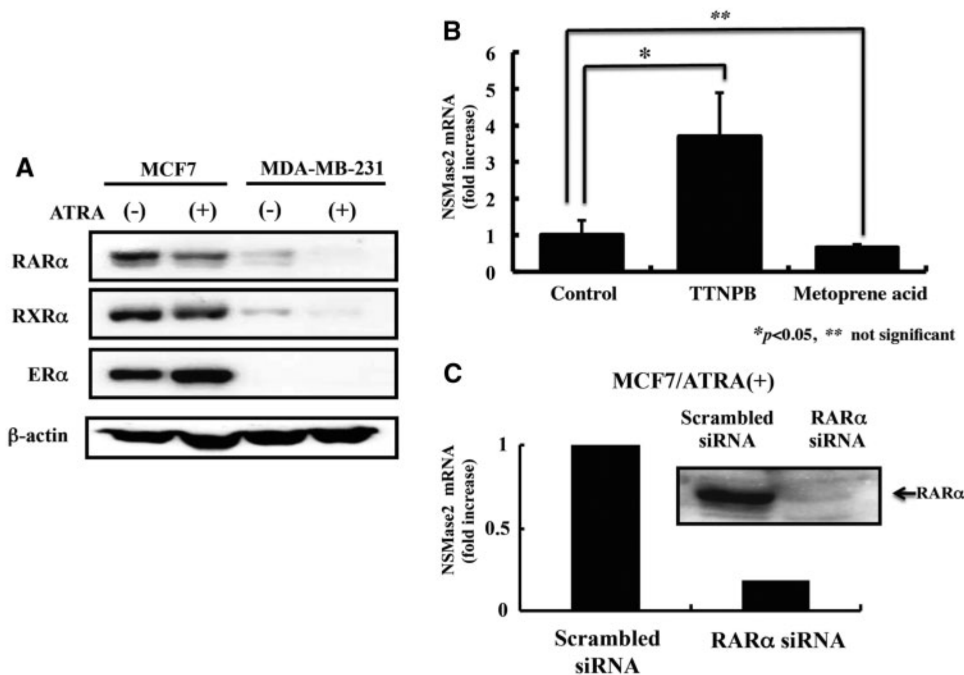

Fig. 3 Expression of RARs and ER. (A) MCF-7 and MDA-MB-231 cells were treated with or without 10 μM ATRA for 24 h. Samples for western blotting were prepared and RARα, RXRα and ERα protein levels were measured using respective antibodies as shown in the 'Materials and Methods' section. (B) RARα and RXRα specific agonists, TTNPB and methoprene acid, were used to examine the change in NSMase2 mRNA levels. TTNPB (10 μM) and methoprene acid (10 μM) were added for 24 h. The mRNA level of untreated MCF-7 cells was shown as the control (regarded as 1.0). Statistical significance was evaluated. (C) siRNAs of RARα and scrambled siRNA were used to examine the effects of RARα silencing on NSMase2 mRNA of ATRA-treated MCF-7 cells. After transfection of respective siRNA, MCF-7 cells were treated with or without ATRA for 24 h. NSMase2 mRNA level was shown. Inlet shows decreased RARα protein level by siRNA of RARα.

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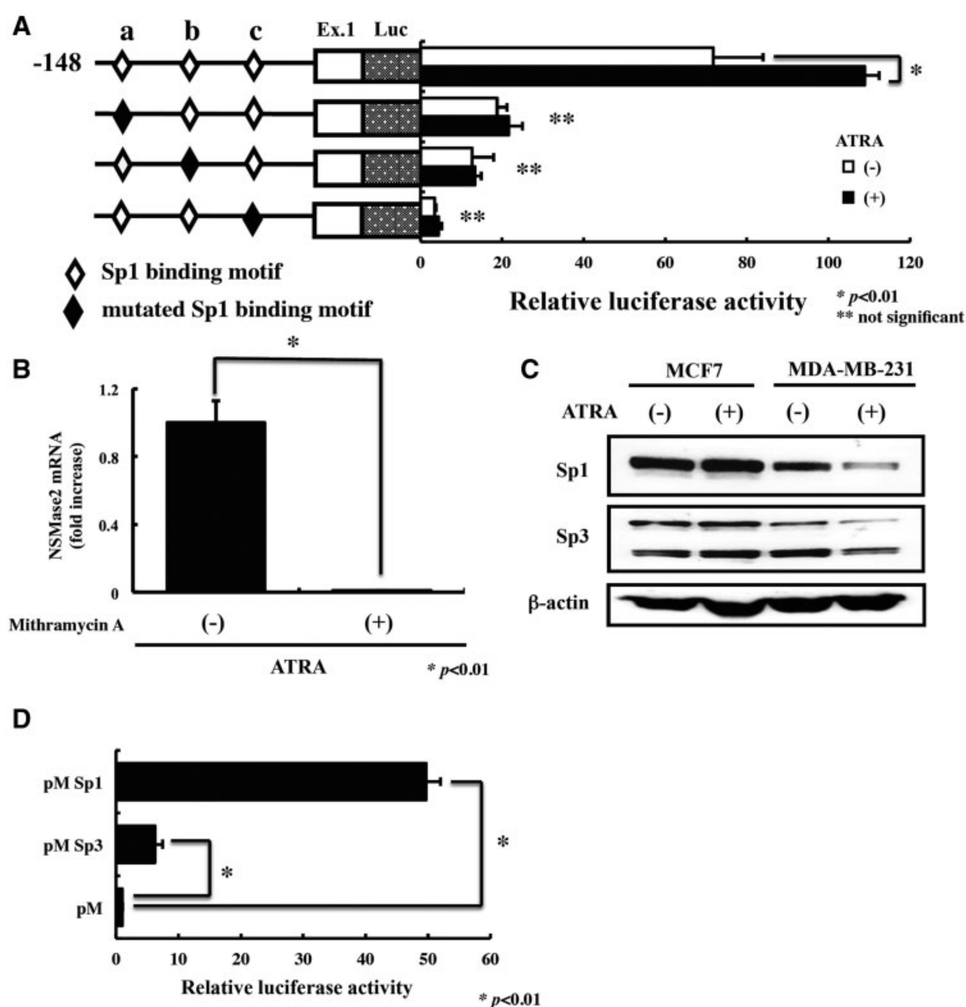

Fig. 4 Promoter analysis of the 5′-promoter region of NSMase2. (A) The promoter region of NSMase2, its deletion constructs and Sp1 site-mutated luciferase vectors were described previously (*29*). Open symbols at left show wild-type Sp1 sites, while solid symbols indicate mutated Sp1 sites. Luciferase assay was performed as described in the 'Materials and Methods' section. Relative luciferase activity was shown as the luciferase activity/β-galactosidase activity. Open columns: ATRA untreated control culture for 24 h and solid columns: ATRA treatment for 24 h after respective reporter vector transfection. Statistical significances were calculated and shown as asterisks. (B) Effect of Mithramycin A was examined in ATRA-treated MCF-7 cells. MCF-7 cells were pre-treated with or without Mithramycin A (500 nM) for 12 h prior to administration of 10 μM of ATRA for 7 h. Cells were collected, and NSMase2 mRNA was measured with the quantitative RT–PCR method as described in the 'Materials and Methods' section. (C) Effect of ATRA on Sp1 and Sp3 protein levels. MCF-7 and MDA-MB-231 cells were treated with or without 10 μM of ATRA for 24 h. Cellular Sp1 and Sp3 levels were analysed by western blotting. (D) Promoter activity of over-expressed Sp1 and Sp3 of ATRA-treated MCF7 cells. MCF7 cells were transfected with pM series expression vector (pM-Sp1, pM-Sp3 or pM), pG5-luc vector and β–gal expression vector according to the 'Materials and Methods' section. After transfection, cells were treated ATRA for 24 h and luciferase activity was measured. The relative luciferase activity of control group (pM) was determined as 1.0.

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GST-Sp1 fusion protein (Fig. 6C). Furthermore, Rottlerin significantly inhibited Sp1 phosphorylation, confirming the involvement of PKCδ in Sp1 phosphorylation (Fig. 6D).

Chromatin immunoprecipitation analysis

Figure 7 shows the results of the Chromatin immunoprecipitation (ChIP) assay. Significant changes of Sp1 and Sp3 were not observed in MCF-7 cells treated with ATRA, which was consistent with our EMSA data (Supplementary Fig. S3). Interestingly, RARα and RXRα were found to be present as a complex bound to this promoter region regardless of ATRA treatment. It was suggested that the RARα and RXRα complexes endogenously bound with Sp1. Acetylated histone H3 but not H4 was observed to increase with ATRA treatment, suggesting ATRA-induced active chromatin configuration. Treatment with the HDAC inhibitor, Tricostatin A, increased NSMase2 mRNA (data not shown). In contrast, in control MDA-MB-231 cells, Sp1 and acetylated histone H3 were found to bind to the 5′-promoter of NSMase2, which is compatible with considerable endogenous NSMase2 mRNA of MDA-MB-231.

Discussion

We previously reported that ATRA-induced ASMase gene expression followed by cellular ceramide increase and cell growth arrest in human NB4 leukaemia cells

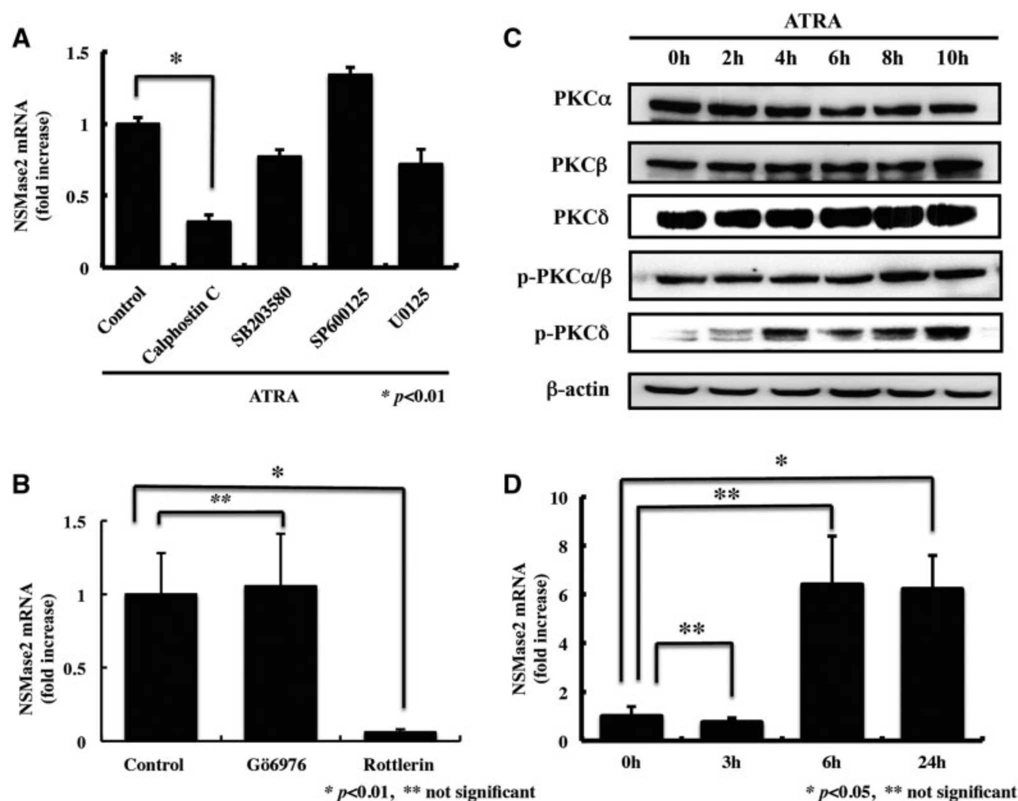

Fig. 5 Effects of signal transduction inhibitors on NSMase2 mRNA. (A) Three hours before ATRA addition, various signal transduction inhibitors, Calphostin C (pan PKC inhibitor; 200 nM), SB203580 (p38 MAPK inhibitor; 5 μM), SP600125 (JNK inhibitor; 20 μM) and U0126 (MEK inhibitor; 10 μM) were added. After ATRA treatment for 21 h, MCF-7 cells were collected and their NSMase2 mRNA was measured by quantitative RT–PCR. (B) PKCα inhibitor, Gö6976 (10 nM) and PKCδ inhibitor, Rottlerin (10 μM), were used to examine their effects on NSMase2 mRNA level. Each inhibitor was added 2 h before administration of 10 μM of ATRA and cultures were continued for another 12 h. NSMase2 mRNA level was measured by quantitative RT–PCR in triplicate with the data of control cells regarded as 1.0. (C) PKC isoforms of MCF-7 cells were examined after ATRA treatment for the indicated times using respective antibody described in the 'Materials and Methods' section. (D) Temporal changes of NSMase2 mRNA of ATRA-treated MCF-7 cells were measured by quantitative RT–PCR as described in the 'Materials and Methods' section.

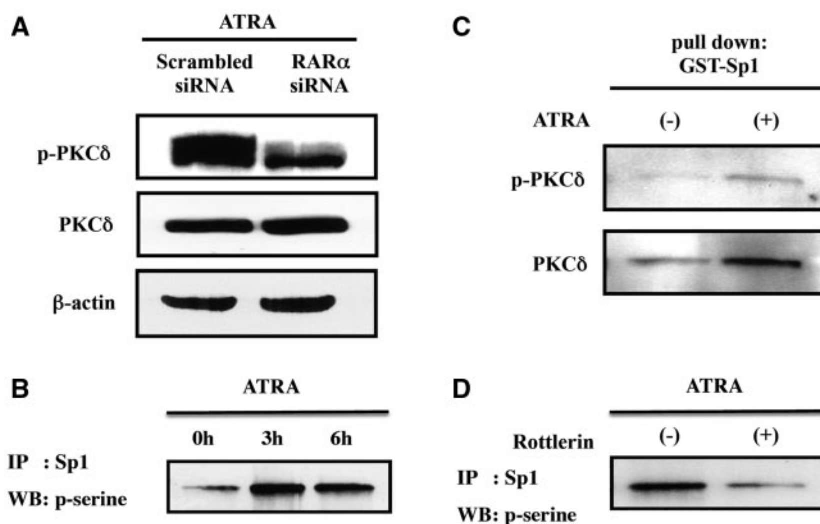

Fig. 6 Relationship between Sp1 phosphorylation and NSMase2 mRNA level. (A) Effect of siRNA of RARα on phosphorylated PKCδ was shown. Twenty-four hours after transfecting siRNA of RARα or scrambled siRNA, MCF-7 cells were treated with ATRA (10 μM) for 24 h. Cells were collected and total and phosphorylated PKCδ were detected by western blotting. (B) Serine phosphorylation of Sp1 induced with ATRA was examined. Cell lysate was immunoprecipitated with anti-Sp1 antibody followed by anti-phosphoserine antibody according to the 'Materials and Methods' section. (C) Binding of partially purified GST-Sp1 and whole-cell lysate of MCF-7 cells with or without ATRA treatment was analysed according to the 'Materials and Methods' section. After washing glutathione particles complexed with GST-Sp1/PKCδ (both phosphorylated and non-phosphorlated form), PKCδ and phosphorylated PKCδ were detected by western blotting. (D) MCF-7 cells were treated with or without Rottlerin for 6 h, then ATRA was added for another 3 h. Phosphorylated Sp1 was detected in the similar way as shown in Fig. 6B.

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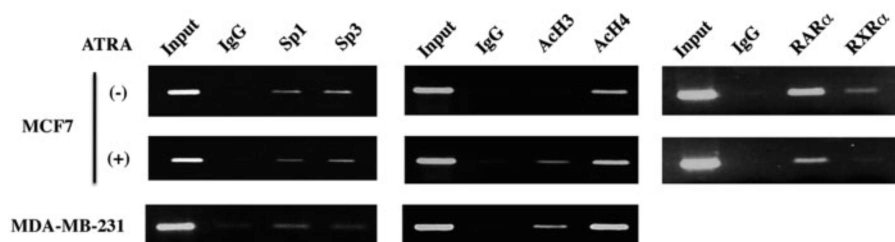

Fig. 7 ChIP assay. MCF-7 cells treated with or without 10 μM of ATRA for 24 h. Control MDA-MB-231 cells were also examined. Antibodies used (anti-Sp1-, anti-Sp3-, anti-acetylated H3-, anti-acetylated H4-, anti-RARα- and anti-RXRα-antibody) were described in the 'Materials and Methods' section. After DNA extraction and protein digestion, PCR was performed using primers described in the 'Materials and Methods' section. The product was 225-bp long.

(*26*). Recently, it has been reported that NSMase2 is at least partially responsible for the growth arrest in ATRA-treated MCF-7 cells through modulation of S6 kinase (*28*). The present study revealed that ATRA increased NSMase2 but not ASMase mRNA as well as cellular ceramide content of a human breast cancer cell line, MCF-7, but not in ATRA-resistant MDA-MB-231 cells. This suggests the possibility that the increased NSMase2 expression by ATRA was related to increased cellular ceramide in MCF-7, although involvement of ASMase cannot be completely neglected (Figs 1 and 2; Supplementary Fig S1). Therefore, ATRA-induced SMase subtype expression is cell type specific.

Although biological effects of ATRA in human breast cancer cells have been studied extensively, the relationship between ATRA and sphingolipid metabolism of breast cancer cells has not been fully disclosed. Experiments using siRNA of RARα and a RAR-specific agonist, TTNPB, suggest that RAR, especially RARα, was prerequisite for ATRA-induced NSMase2 mRNA elevation of MCF-7 cells (Fig. 3B and C). Nuclear run-on assay as well as mRNA half-life measurement (Fig. 2C and Supplementary Fig. S2) demonstrated that the increased transcription is mostly responsible for the ATRA-induced elevation of NSMase2 mRNA level, although the minor contribution of mRNA half-life elongation cannot be neglected.

ERα, RARα and cellular retinoic acid-binding protein are co-ordinately regulated (*13*). In preliminary experiments, we have consistently over-expressed ERα in MDA-MB-231 cells that originally lack ERα, RARα and RXRα expression (Fig. 3A). However, these ERα-over-expressed cells did not show the recovery of RARα and RXRα expression as well as ATRA-sensitivity (data not shown). Thus, ERα re-expression is not sufficient to induce RARα and RXRα expression or the expression of these genes is independent.

Indirect involvement of the RARα/RXRα complex is most likely in ATRA-induced NSMase2 mRNA transcription of MCF-7 cells, because the 5′-promoter region of NSMase2 lacks consensus RAREs. We recently reported the importance of Sp1 sites between −148 and −42 bp from the first exon in both endogenous and daunorubicin-induced NSMase2 transcription of MCF-7 cells and also showed that Sp1 protein was endogenously bound to several Sp1 sites of the 5′-promoter region of NSMase2 (*29*). Other studies have reported Sp1-induced activation in ATRA-enhanced gene expression in different cell types (*20*, *34*), and our promoter analysis (Fig. 4A) indicates that the same region is also important for NSMase2 transcription of ATRA-treated MCF-7 cells. However, the increased promoter activity by ATRA was much less than expected. Although other promoter or enhancer regions might increase NSMase2 transcription, increase of acetylated Histone 3 around this promoter region by ATRA (Fig. 7) suggested the significant involvement of chromatin remodelling rather than enhanced transcription factor binding, which can be not efficiently detected by the luciferase reporter assay. Among Sp family proteins, Sp1 rather than Sp3 is the major transcription factor to regulate NSMase2 transcription (Fig. 4D).

Not all RA-inducible genes contain RARE/RXRE sequences within promoter (*35*), and VEGF gene expression is induced by retinoic acid through Sp1-binding sites (*34*). The physical interaction between RARα and Sp1 has been reported (*36*), and RARα/RXRα tethers with Sp1 protein without direct binding to RAREs (*37*). Our EMSA assay (Supplementary Fig. S3) did not demonstrate the direct binding of RARα and RXRα to this promoter DNA; however, ChIP assay (Fig. 7) suggested the complex formation of RARα/RXRα with Sp1 protein. Thus, it was assumed that RARα and RXRα were bound to this promoter region indirectly through binding to Sp1 protein. Similarly, it has been recently reported that RARα/RXRα was endogenously bound to the 5′-promoter region of guanylyl cyclase/natriuretic peptide receptor-A indirectly by the complex formation with Sp1 in mouse mesangial cells (*38*).

The use of a Sp1-specific inhibitor, Mithramycin A, confirmed involvement of the Sp family protein in ATRA-induced NSMase2 transcription (Fig. 4B). ATRA did not modulate total Sp1 protein level nor its DNA-binding capacity (Fig. 4C and Supplementary Fig. S3). Thus, Sp1 protein modification such as phosphorylation was a highly plausible mechanism of ATRA-induced NSMase2 transcription. Increased Sp1 phosphorylation has been shown to be a possible mechanism of HGF-induced VEGF transcription (*39*). The treatment with HGF increased serine phosphorylation of Sp1 leading to increased transcription activity

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of Sp1 without affecting its DNA-binding profile of EMSA. Actually, serine-phosphorylated Sp1 was observed in ATRA-treated MCF-7 cells (Fig. 6B). Our EMSA experiments using protein phosphatase, CIP, revealed that CIP significantly inhibited Sp1 phosphorylation, suggesting the positive involvement of binding of phosphorylated Sp1 protein induced by ATRA to this promoter region (Supplementary Fig. S3d).

Involvement of PKCδ in NSMase2 transcription has also been reported in prostate cancer cells (*40*), but its mechanism remains to be disclosed. As shown in Fig. 5A and B, pharmacological pan PKC- and PKCδ-inhibitors, Calphostin C and Rottlerin, inhibited ATRA-induced NSMase2 transcription. Treatment with ATRA has been shown to activate PKCα or δ in various cell lines, and the complex formation between RARα/RXRα and PKCδ was presumed to occur (*16*, *41–45*). Moreover, our experiments using siRNA of RARα revealed the involvement of RARα for both ATRA-induced PKCδ activation and increased NSMase2 mRNA expression (Figs 3C and 6A). It is thus of note that ATRA-induced events of PKCδ phosphorylation, Sp1 phosphorylation and NSMase2 transcription were sequentially well correlated. Sp1 has been reported to be phosphorylated by a number of cellular kinases including different members of the PKC family (*46*). Some studies have shown the physical interaction of PKCδ and Sp1 (*47*, *48*), which was confirmed by our study using GST-Sp1 protein (Fig. 6C). Furthermore, we have demonstrated that PKCδ is at least partially involved in Sp1 phosphorylation, because Rottlerin reduced Sp1 phosphorylation (Fig. 6D).

In the ChIP assay, ATRA was shown to increase acetylated histone H3 around the promoter region of NSMase2, and the increased histone acetylation indicated the active configuration of this promoter region for the transcription (Fig. 7). ATRA-induced histone acetylation has also been reported in mouse mesangial cells (*38*). Importance of histone remodelling was supported by our preliminary experiments showing increased NSMase2 mRNA by TSA, a HDAC inhibitor.

Based on these results, we proposed a unique transcription mechanism in MCF-7 cells: ATRA-induced PKCδ activation followed by Sp1 phosphorylation, and then phosphorylated Sp1 was bound to the NSMase2 promoter. These sequential events result in the increased transcriptional activity of Sp1 and also remodelled the chromatin configuration of this promoter region to the appropriate structure for active transcription. An important and interesting future project remains to elucidate the signalling pathway from activated PKCδ leading to nuclear histone H3 acetylation.

Taken together, the present study revealed that ATRA sensitivity of human breast cancer cells is associated with the cellular ceramide level and NSMase2 activity. In ATRA-treated MCF-7 cells, NSMase activity was mostly due to increased NSMase2 transcription via phosphorylated Sp1 caused by PKCδ activation. However, the expression of ER *per se* in ATRA-resistant cell line could not recover ATRA sensitivity, suggesting that RAR and RXR expression are not always controlled by ER expression level. Since the novel treatment of human breast cancer is in great demand for ATRA-resistant cases and because the heterogeneity of ATRA sensitivity in breast cancer cells remains to be fully evaluated, the findings obtained in the present investigation provide useful information for understanding the relationship between ATRA sensitivity and the sphingolipid metabolism in breast cancer cells.

Supplementary data

Supplementary data are available online at *JB* online.

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Conflicts of interest

None declared.

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