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Transcriptional regulation of neutral sphingomyelinase 2 gene expression of a human breast cancer cell line, MCF-7, induced by the anti-cancer drug, daunorubicin

(ヒト乳癌細胞株 MCF-7 における抗癌剤ダウノルビシン による中性スフィンゴミエリナーゼ2 遺伝子発現の転写調節機序)

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主論文の要旨

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[緒言]

近年提唱されているスフィンゴ脂質レオスタットモデルでは、細胞内のスフィンゴシン 1-リン酸／セラミドの比率が細胞の成長、細胞死、分化などの細胞の運命を決めるとされる。スフィンゴミエリナーゼ (SMase)は膜に存在するスフィンゴミエリン(SM)を分解し、セカンドメッセンジャーとして働くセラミドを産生する。セラミドはアポトーシスを誘導する重要な分子であると考えられている。

SMases は最適 pH の範囲などによって分類され、現在では５つのタイプの SMase が存在する。その中でも主にリソソームにある acid SMase (ASMase)と Mg^{2+}依存性の neutral SMase (NSMase) がストレス誘導性のセラミド産生に働いていると考えられている。

NSMase2 を過剰発現すると SM 代謝に変化が引き起こされたり、細胞をコンフルエントにすると NSMase 活性や NSMase2 mRNA の発現が増加したりする。NSMase2 ノックアウトマウスでは、野生型のマウスより成長が遅れること等が報告されている。今回の実験では抗癌剤ダウノルビシンによって誘導される NSMase2 遺伝子発現増加の調節機序について調べることにした。

[対象及び方法]

ヒト乳癌細胞株 MCF-7 細胞を用いて以下の実験を行った。アポトーシスの判定には caspase3 colorimetric protease assay kit を用いた capsase 3 活性の測定、Annexin V を用いた細胞数の算出により行った。セラミド量の測定には、LC/MS-MS を使用し、NSMase 酵素活性測定には、[N-methyl-^{14}C] sphingomyelin を用いた。mRNA 量は Real time PCR を用いて測定し、タンパク量は Western Blotting を行った。NSMase2 過剰発現は、3'-Flag tag 付き mouse NSMase2 発現ベクターを用いて MCF-7 細胞にて安定発現株を作製した。転写調節について NSMase2 promoter 解析は Luciferase assay を行った。Electrophoresis Mobility Shift Assay (EMSA)ではビオチン標識した DNA プローブを作製し、実験を行った。Chromatin immunoprecipitation assay (ChIP assay) では、３つの Sp1 結合モチーフを含むようにプライマーを設定した。

[結果]

MCF-7 細胞を DA で刺激すると、アポトーシスが誘導されることを caspase3 活性の増加、annexin V 陽性細胞の増加により確認した。この条件下でセラミド量、酵素活性を測定したところ DA によって細胞内セラミド量、NSMase 酵素活性の増加を認めた。DA で刺激した時の NSMase2 mRNA、NSMase2 タンパク質についても DA 刺激時に発現が増加した。

NSMase2 を過剰発現、または抑制した時の DA 刺激に対する細胞の抵抗性について調べた。NSMase2 過剰発現株では mock に比べて生存率が低下していた。一方、NSMase2 の発現を抑制すると、生存率の低下が見られなくなった。

次に様々な長さのプロモーターコンストラクトを作製し、NSMase2 5’ プロモーターの解析を行った。DA 刺激により NSMase2 プロモーターの活性が増加し、さらに転写開始点を+1 として-148bp～-42bp のところで活性が大きく変化したため、この領域に基本転写活性があることが推測された。この領域には 3 つの Sp1 結合モチーフが存在する。Sp1 結合モチーフに変異を入れたコンストラクトを作製し、プロモーター解析を行ったところ、活性の低下が認められた。 Sp family の阻害剤である mithramycin A や Sp1 siRNA、Sp3 siRNA を用いて Sp1、Sp3 の発現を抑制したところ、いずれも NSMase2 mRNA 発現が抑制された。これらの結果から Sp1、Sp3 が DA 刺激による NSMase2 の発現の増加に関与していることが明らかとなった。

さらに 3 つの Sp1 結合モチーフにプローブを設定して EMSA を行った。DA 刺激により、バンドの増強が認められた。抗 Sp1 抗体、抗 Sp3 抗体を用いてスーパーシフトを行ったところ、Sp1、Sp3 が結合することが明らかになった。in vivo でのこれらのプロモーター領域への結合について解析するために、この 3 つの Sp1 結合モチーフを含むように設定したプライマーを用いて ChIP assay を行った。抗 Sp3 抗体では DA 刺激の有無にかかわらず結合が確認できたが、抗 Sp1 抗体では DA 刺激により結合の増強が認められた。

[考察]

MCF-7 細胞において DA 刺激により、NSMase２ mRNA、タンパク質の発現が増加し、細胞内セラミド、NSMase 酵素活性も増加することが明らかとなった。さらに NSMase2 の発現が増加すると DA の感受性が増加し、NSMase の発現が低下すると DA の感受性も低下することが明らかになった。SMase にはいくつかのアイソザイムが存在するが、mRNA の発現レベルや酵素活性の比較により、DA 刺激によるセラミド産生の増加には NSMase2 の役割が最も重要であることが示唆された。

DA 刺激による NSMase2 の発現の増加について mRNA の転写レベルで解析した結果、転写因子 Sp1 の関与が認められた。通常、NSMase2 のプロモーターには Sp1、Sp3 が結合しているが、DA 刺激により Sp1 の NSMase2 プロモーターへの結合が増強し転写を促進することが明らかとなった。

[結語]

DA 処理により、NSMase2 mRNA は他の SMase よりも顕著に発現が増加することが明らかになった。またその調節機序の一つとして、細胞内に普遍的に存在している転写因子 Sp ファミリーが NSMase2 転写調節に関与していて、抗癌剤 DA が転写因子 Sp1 と NSMase2 5’ プロモーターとの結合を増強させ、転写を促進することにより NSMase2 mRNA の発現が増加することが明らかとなった。

主論文

ABSTRACT

Mg^{2+}-dependent neutral SMases (NSMases) have emerged as prime candidates for stress-induced ceramide production. Among isoforms identified, previous reports have suggested the importance of NSMase2. However, its activation mechanism has not been precisely reported. Here, we analyzed the mechanism of *NSMase2* gene expression by the anti-cancer drug, daunorubicin (DA). DA increased cellular ceramides (C16, C18 and C24) and NSMase activity of a human breast cancer cell line, MCF-7. DA remarkably increased the *NSMase2* message and protein, whereas little change in *NSMase1* and *NSMase3* mRNA and only a mild increase in *acid SMase* mRNA were observed. Overexpression and a knock down of *NSMase2* indicated that NSMase2 played a role in DA-induced cell death. NSMase2 promoter analysis revealed that three Sp1 motifs located between -148~-42 bp upstream of the first exon were important in basic as well as in DA-induced promoter activity. Consistently, luciferase vectors containing three consensus Sp1-motifs but not its mutated form showed DA-induced transcriptional activation. DA-treated MCF-7 showed increased Sp3 protein. In SL2 cells lacking Sp family proteins, both Sp1 and Sp3 overexpression increased *NSMase* promoter activity. Increased binding of Sp family proteins by DA to three Sp1 motifs was shown by electrophoresis mobility-shift and ChIP assays.

Keywords: NSMase2, Daunorubicin, Ceramide, Promoter analysis, EMSA, ChIP,
Sp family protein

Abbreviations: SMase, sphingomyelinase; SM, sphingomyelin; EMSA, electrophoresis mobility shift assay; ChIP, chromatin immunoprecipitation; DA, daunorubicin; Sp, specificity protein;

Introduction

Spiegel et al. [1] proposed the sphingolipid rheostat model, where cellular sphingosine 1-phosphate/ceramide ratio determines a cell's fate in areas such as cell growth, cell death, differentiation and cell movement. Sphingomyelinase (SMase) hydrolyzes the membrane sphingomyelin (SM) to ceramide, which is considered to serve as the second messenger. Cellular ceramide then undergoes further metabolism. It could be metabolized to sphingosine, sphingosine 1-phosphate, or other glycosyl sphingolipids. Therefore, ceramide is found to be located at the central part of sphingolipid metabolism [2], and has also been recognized as an important player in the induction of apoptosis [3].

Though the kinetics of SM hydrolysis varies depending on stimuli and cell types, two types of response can be discerned. In the vast majority of cases, stress-induced SM hydrolysis occurs rapidly within minutes [4]. On the other hand, some cytotoxic agents induce a late hydrolysis of SM and ceramide accumulation over a period of several hours or days, suggesting that the SM-ceramide pathway fulfills some function in the execution phase of apoptosis.

SMases have been classified by their optimal pH range. Currently, five types of SMases have been identified; an ubiquitous lysosomal acid SMase, a zinc-dependent secreted acid SMase, a neutral Mg^{2+}-dependent SMase, a neutral Mg^{2+}-independent SMase and an alkaline SMase [5]. Of these, the lysosomal acid SMase and the Mg^{2+}-dependent neutral SMase have emerged as the prime candidates for stress-induced ceramide responses.

Three *NSMases* have been cloned. Among the *NSMases*, *NSMase1* overexpression showed no changes in SM metabolism, whereas it was shown to act as a lyso-PAF phospholipase C in vivo rather than as a NSMase. In fact, it favored lyso-PAF acting as a preferred substrate in vitro [6]. In contrast, overexpression of *NSMase2* was shown to cause a change in SM metabolism, especially an increase in very long chain ceramides, concurrent with a decrease in SM [7]. Furthermore, it has been reported that cellular confluence induced an up-regulation of NSMase activity and *NSMase2* mRNA [8]. *NSMase2* knockout mice showed no deficiency in lipid storage but did exhibit as much as 50% less growth than the wild type, which remained throughout their development [9]. We reported the decreased *NSMase2*

message in the bone marrow of myelodysplastic syndromes and acute leukemia compared with normal control bone marrow [10]. Moreover, mutations in *NSMase2* were reported in some leukemia patients [11], suggesting that these changes in the *NSMase2* message are related to leukemogenesis and/or chemosensitivity. *NSMase3*, which shares very little homology with *NSMase2*, has been cloned very recently [12], but its pathophysiological role remains to be determined.

In the current study, we focused on the regulatory mechanism of *NSMase2* gene expression by DA treatment. We previously reported the regulatory mechanism of *ASMase* gene expression induced by all-trans retinoic acid (ATRA) [13]. It is thus of considerable interest to compare the gene expression mechanism between *ASMase* and *NSMase2*, both of which are regarded as two major SMases in the apoptosis process. Our analysis clearly suggested that *NSMase2* is more sensitive to the transcriptional regulation by an anti- cancer drug of DA than other *SMases*. We have, for the first time, provided basic information about the regulatory mechanism of *NSMase2* gene expression, which might prove important for furthering our understanding of the apoptotic process induced by anti-cancer drug treatment.

Material and methods

Cell lines and reagents

A human breast cancer cell line, MCF-7, was cultured in Dulbecco's Modified Eagle's medium (Sigma, St. Louis, MO, USA) with 5% fetal calf serum (JRH Biosciences Inc. Lenexa, KS, USA) at 37°C in 5% CO_2. A drosophila cell line, SL2, which lacks Sp series transcription factors, was the generous gift of Prof. T. Noguchi (Osaka Otani University, Osaka, Japan). SL2 cells were cultured in Schneider's medium (Invitrogen, Carlsbad, CA, USA) with 10% FCS. Daunorubicin (DA) was purchased from Sigma; Mithramycin A from Fulka (Buchs, Switzerland); pGL3 and pGL4 basic vectors for luciferase assay were from Promega (Madison, WI, USA). siRNA of human *NSMase2* (*hNSMase2*) was purchased from Sigma Genosys (Hokkaido, Japan). The sequence of siRNA of *hNSMase2* was according to Marchesini *et al.* [8]. siRNA of human *Sp1* and *Sp3* were prepared with the sequence of 5'- GGAUGGUUCUGGUCAAAUATT-3', 5'-GUUGGGGGAGGUGGAGCCUTT-3', respectively [14]. Non-targeting siRNA #1 (Dharmacon) was used as the control scramble siRNA. Sp series expression vectors for SL2 cells, pPac, pPac-Sp1 and pPac-USp3 [15] were originally derived from Prof. G. Suske (Philipps-Universitaet Marburg, Marburg, Germany). For correction of the transfection efficiency of SL2 cells, pPac-RL was used as previously described [16]. Sp1TransLucent reporter vector (Sp1(2)), which is designed to monitor transcription factor-binding activity of the Sp family through the use of a standard luciferase assay, was purchased from Panomics (Redwood City, CA, USA). Flag tagged mouse *NSMase2* expression vector was derived from Dr. YA. Hannun (University of South Carolina, SC, USA).

Western blotting

Western blotting was performed using anti-human NSMase2 antibody (x500 dilution, H-195, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti b-actin antibody (x2000 dilution, Cytoskeleton Inc., Denver, CO, USA) and anti-FLAG antibody (x1000 dilution, Sigma), respectively. Anti-Sp1 (x1000 dilution, PEP2, Santa Cruz) and anti-Sp3 antibody (x1000 dilution, D-20, Santa Cruz) were also used. ECL plus Western blotting system (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK) was

used throughout the experiments.

Measurement of viable cells and the analysis of apoptosis

MCF-7 cells were plated in a tissue culture plate of 35 mm in diameter ($3.0x10^5$/ml) in triplicate 24 h before 0.5 μM DA or other reagents were added. For the detection of apoptotic cells, Annexin–V-FLUOS test (Roche Diagnostics, Indianapolis, IN, USA) were used to detect apoptotic cells (surface phosphatidyl serine (+)) according to the manufacturer's manual. Propidium iodide (final concentration: 1 μg/ml) was also used simultaneously to detect the late apoptotic cells by labeling the nuclei. Stained cells were observed under fluorescent microscopy. Annexin-V positive cells were counted as apoptotic cells. At least 300 cells were counted and the percentage of positive staining was calculated. Viable cell numbers were counted using the trypan blue dye exclusion test. Viable cell numbers on day 0 were regarded as 100%.

Caspase 3 enzyme activity was measured using caspase 3 colorimetric protease assay kit (Medical and Biological Laboratories CO., LTD, (MBL), Nagoya, Japan). The assay is based on spectrophotometric detection of the cleaved product of DEVD-p-nitroanilide, and was measured as the light emission of 400 nm using the microplate reader according to the manual of the manufacturer. The data of control MCF-7 cells were determined as 1.0.

Real-time and semi-quantitative RT-PCR

Total RNA was extracted using the RNeasy mini kit (QIAGEN, Germantown, MD, USA). The first strand cDNA was prepared with 5 μg of RNA using the Super Script III First Strand System (Invitrogen, Carlsbad, CA, USA). Quantitative PCR was performed with the Power SYBR (Applied Biosystems, Foster City, CA, USA) in duplicate according to Sobue *et al.* [10]. ABI PRISM 7000 sequence detection systems (Applied Biosystems) were used for the measurements. The primer sequences were shown in Table 1. In addition to GAPDH, b-actin primer set was also used as another internal control. The PCR condition of NSMase2 and GAPDH was 50℃ 2 min, 95℃ 10 min, followed by 40 cycles at 95℃ for 15 sec and 63℃ for 45 sec. Those of *ASMase, NSMase1* and *NSMase3* were the same except for the extension temperature (64℃ for *ASMase*, 62℃ for *NSMase1* and 60℃ for *NSMase3* and *b-actin*). Semi-quantitative RT-PCR was performed with LA *Taq* (Takara

Bio Co, Shiga, Japan) for NSMase2 and KOD FX *Taq* (Toyobo Biochemical Co, Osaka, Japan) for GAPDH, respectively. Primer sets and annealing temperature used were shown in Table 1 and the PCR cycles were illustrated Figure 5c.

Acid and neutral SMase enzyme activities

Cells were sonicated in 50 mM HEPES-NaOH buffer (pH 7.5) containing a protease inhibitor mixture (Complete-mini, EDTA-free)(Roche Molecular Biochemicals) and 0.5 mM DTT. The assay mixture for the determination of NSMase activity contained 0.1 M Tris/HCl (pH 7.5), 0.1 mM DTT, 10 mM $MgCl_2$, 0.05% Triton X-100, 1.2 M KCl, 20 μM phosphatidylserine, 20 μM [N-methyl-^{14}C] sphingomyelin (20,000 cpm, adjusted by cold sphingomyelin) and 5~10 μg protein of cell homogenate for a total of 50 μl. After incubation for 30 min at 37°C, the radioactivity of released phosphocholine was determined by a liquid scintillation counter. The assay of ASMase has been described previously [13]. The assay mixture for ASMase contained 0.1 mM acetate buffer, pH 5.8, 1 mM EDTA, 22 mM [N-methyl-^{14}C] sphingomyelin, 0.05% TritonX-100, 1.2 mM KCl and 10~20 mg protein of cell homogenate for a total of 50 μl.

Cellular ceramide measurement

Cellular lipid was extracted by a method originally described by Bligh and Dyer [17], and cellular ceramide was measured by the LC-MS/MS method using a Waters 2695 HPLC system (Milford, MA, USA) coupled to a Micromass Quatromicro API triple-quadrapole mass spectrometer (Micromass, Manchester, UK) as described previously [18]. The mass spectrometer was operated using an electrospray atmospheric pressure ionization (ESI) source in positive ion mode with multiple reaction monitoring (MRM). Chromatographic separations were obtained under gradient conditions using a Cadenza HS-C18 column (Imtakt Co., Kyoto). The mobile phase consisted of solvent A (water/ trifluoroacetic acid (TFA), 100:0.1 v/v), solvent B (acetonitrile/TFA, 100:0.1 v/v) and solvent C (acetone/TFA, 100:0.1 v/v). A standard curve was made using purified C16, C18 and C24 ceramide.

Cloning of hNSMase2 and hNSMase1 promoter regions

The -984 bp /+109 bp region of hNSMse 2 was obtained by PCR methods with PrimeSTAR HS DNA polymerase (Takara Bio Co, Shiga, Japan) using primers 1 and 2 (Supplementary information 1). It was digested by *Xba*I and *Hin*dIII and inserted to the pGL3 basic vector. The -319 bp/+109 bp region was obtained by PCR using primers 3 and 2. It was inserted at *Xho*I and *Hin*dIII sites of pBluescript II KS (+) cloning vector. Next, the -319 bp/+109 bp Luc was produced by inserting at the *Kpn*I and *Hin*dIII sites of the pGL3 basic vector. The other truncated forms except -81 bp were also obtained by PCR methods using specific forward primers and GL primer2 and digested by *Nco*I. The respective fragments were inserted into the *Sma*I (blunt end) and *Nco*I of the pGL3 basic vector. The -81 bp/+109 bp Luc was obtained from the -319 bp/+109 bp Luc that was digested by *Kpn*I and *Bst*EII, blunted, and ligated. The sequences of all constructs were confirmed.

Mutations were inserted into 3 Sp1 binding motifs as shown in Figure 4 by overlap extension PCR methods [19] with the -148 bp/+109 bp Luc as the template. The methods and primer sets used were illustrated in the supplementary information 2. Two DNA fragments whose DNA sequences overlapped were obtained by the first PCR. In (a) of the supplementary Fig. 2, for example, two PCR fragments (a product of primer A/GL primer2 and a product of RV primer 3/primer A') were used for the second overlap extension PCR with primer set (RV primer 3 and GL primer 2). The final PCR product was digested by *Sac*I and *Nco*I, and it was inserted into the *Sac*I and *Nco*I site of the pGL3 basic vector.

We also obtained an approximately 1.0 kb fragment covering the 5' region of exon 1 of *hNSMase1 (SMPD2:* NM_003080*)* by a PCR method using the following primers;
forward 5'-GGGATTACAGAGACCGCCACCA-3',
reverse 5'-TCCCGGTTCCTTTTCCTCCA-3'.
The fragment was inserted into the *Eco*RI and *Eco*RV sites of pBluescript KS (+). The -938 bp/+81 bp Luc was then produced by inserting it into the *Sac*I and *Hind*III sites of the pGL3 basic vector.

Luciferase assay

DNA transfection was performed by the calcium phosphate precipitation method. Five μg of various lengths of luciferase vector and 2 μg of β-galactosidase expression vector were cotransfected to MCF-7 cells ($3x10^5$

cells/plate) cultured in a tissue culture plate with the diameter of 35 mm. Cells were treated with 10% glycerol in DMEM for 1 min 6 h after transfection as described previously [20]. The cells were further cultured in the medium with or without 0.2 μM daunorubicin for another 36 h. Luciferase and β-galactosidase activities were measured, and the relative promoter activity was expressed as luc/β-galactosidase activity. ASMase promoter was as previously described [13].

siRNA treatment

siRNA of *NSMase2, Sp1, Sp3* or a scrambled siRNA was transfected to MCF-7 cells by either Oligofectamin (Invitrogen) or lipofectamin 2000 (Invitrogen) as described previously [18].

Sp1 Translucent reporter assay

The Sp1TransLucent reporter vector (Sp1 (2)) is designed to monitor Sp family transcription factor-binding activity. MCF-7 cells with or without DA treatment were transfected with 3 μg of either control TransLucent reporter vector or Sp1 (2) vector by the calcium phosphate precipitation method. To normalize the promoter activity, 2 μg of β-galactosidase expression vector was cotransfected. Reporter activity was expressed as described above.

Transfection to SL2 cells

SL2 cells ($1x10^6$ cells/plate) were cultured in a tissue culture plate of 35 mm in the diameter. Transfection of pPac series expression vector (50 ng) together with 2 μg of -148 bp NSMase2/luc vector was performed by the calcium phosphate precipitation method as described above. When luciferase activity was measured together with pPac-Sp in SL2 cells, the core of the luciferase vector, pGL3 basic vector, was replaced with pGL4 basic vector (Promega). Relative reporter activity was calculated as pPac-Sp/pPac-RL.

Electrophoresis mobility shift assay (EMSA)

Nuclear extract was prepared from MCF-7 cells with or without DA treatment for 24 h. EMSA was performed according to the method previously described [21]. Labeled wild type oligo probe *a,* mutated probe *a,* wild type probe *b,* wild type probe *c and* mutated probe *c* were shown in Table 2. Mutated nucleotide was underlined. A supershift assay was conducted using

2 μg of either anti-Sp1 or anti-Sp3 antibody (Santa Cruz), respectively.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as described previously [21]. For the immunoprecipitation, anti-Sp1, or anti-Sp3 antibody (Santa Cruz, 200 ng/sample) was added and incubated overnight at 4°C. Normal rabbit IgG was used as a control IgG. After DNA extraction, the promoter region containing a Sp1 motif was amplified by PCR using the following primers, forward 5'-GGTCTCTGGGGGATGTGGTCTTG-3'; and reverse 5'-GGCTCTCGCGGCTCTCGGGT-3'. The size of the PCR product was 225 bp. PCR was performed using a KOD FX polymerase (Toyobo Biochemical) and PCR condition was 94°C for 2 min followed by 35 cycles of 98°C at 15 sec, and 68°C at 45 sec.

Statistical analysis

Statistical analysis (Student's *t* test) was performed with Microsoft Excel and its affiliated software.

Results

Cellular ceramide level and NSMase activities after DA treatment

MCF-7 cells have been reported to show apoptotic changes by the anti-cancer drug, daunorubicin [22, 23]. We repeated these results by analyzing caspase3 enzyme activation and annexin V positive cells. Fig. 1a shows caspase3 activation by DA. Fig. 1b illustrates that viable MCF-7 cells decreased by DA treatment for 48 h and that DA increased (surface annexin V positive) apoptotic cells, as compared with the control cells without DA-treatment (Day 0). In order to study the sphingomyelinase (SMase) in the process of apoptosis, we measured cellular ceramide and sphingomyelinase enzyme activity. Fig. 1c shows the change of cellular ceramide levels after DA treatment. All the C16, C18 and C24 ceramides were increased 24 h after 0.5 μM of DA treatment. In parallel, neutral SMase (NSMase) activity was increased almost two-fold by DA (Fig. 1d). Acid SMase (ASMase) also increased about 50% after 24 h of DA treatment.

Changes of hNSMase2 expression by various cellular stresses

To examine whether hASMase or hNSMase was responsive to the stress-response pathways, MCF-7 cells were treated with DA or cultured for 48 h after cells reached the confluent state, and the mRNA levels of *hASMase, hNSMase1*, *hNSMase2* and *hNSMase 3* were examined by real time RT-PCR. Among them, *hNSMase2* message was significantly increased by DA, with *hASMase* mRNA also showing a mild increase by DA (Fig. 2a). Under confluent conditions, MCF-7 cells also showed a higher *hNSMase2* mRNA level compared to non-confluent (control) cells. In contrast, *hNSMase 1* and *3* showed no discernible changes. Because DA treatment caused the highest increase in *hNSMase2* mRNA, we focused on NSMase2 with DA treatment in the following experiments. Our preliminary experiments using another human cell line, K562 treated with DA also showed the increase of *NSMase2* mRNA but not *NSMase1* and *NSMase3* mRNA compared with control K562 cells (data not shown). The temporal changes of *NSMase2* mRNA following DA treatment were illustrated in Fig. 2b. DA also increased NSMase2 protein (Fig. 2c). Furthermore, a mild increase in NSMase2 protein was also observed under the confluent condition where cell proliferation was stopped but no apoptosis was observed. We also examined

other anti-cancer drugs, ara-C and camptothecin (CPT). CPT increased *NSMase2* mRNA but ara-C which has been reported to induce NSMase activity [24], induced NSMase2 mRNA very mildly (data not shown), suggesting that *NSMase2* gene expression by anti-cancer drugs is drug-dependent.

Effect of mouse NSMase 2 overexpression and siRNA of hNSMase2 on DA-sensitivity of MCF-7 cell

We established MCF-7 cells that stably overexpressed FLAG-tagged mouse NSMase2 (mNSMase2), while the presence of FLAG-tagged mNSMase2 was confirmed by western blotting using anti-FLAG antibody (Fig. 3a). The increase in NSMase activity of mNSMase2-overexpressed MCF-7 cells was also confirmed. *mNSMase2*-overexpressed MCF-7 cells were more sensitive to DA than mock-MCF-7 cells (Fig. 3b). To further examine the role of NSMase2 in DA sensitivity, *hNSMase2* siRNA was used to inhibit *hNSMase2* expression. Figure 3c showed the inhibition of *hNSMase2* mRNA expression by *hNSMase2* siRNA but not by a scrambled siRNA. Viable cell numbers after DA treatment were higher in *hNSMase2* siRNA-treated cells than in scrambled siRNA-treated cells, suggesting the involvement of *NSMase2* expression levels in the process of DA-induced apoptosis (Fig. 3d).

Promoter analysis of hNSMase2 gene

The promoter assay using luciferase vectors was performed to examine regions responsible for the basal and DA-induced *hNSMase2* gene expression using various truncated vectors as shown in Fig. 4. DA-induced promoter activity was changed considerably between -165 bp and -42 bp (Fig. 4a and b). Available online data of the putative binding motif of the transcription factors of this region contained three putative Sp1 motifs. Taking this information into account, we further dissected the region and found that the major promoter activity occurred between -148 bp and -42 bp (Fig. 4b left). By introducing mutations into the respective Sp1 binding motif (shown as a solid diamond), the proximal Sp1 motif was found to be the most potent among these three Sp1 motifs, and that the combination of all three mutations showed a severe loss of promoter activity (Fig. 4c). Furthermore, we also examined the -519 bp/+300 bp *hASMase* luciferase reporter [13] and

the -938 bp/+81 bp luc of *NSMase1* 5'promoter for their DA sensitivity. DA showed no increased transcription activity of *hASMase* gene promoter, and rather decreased hNSmase1 gene promoter (Fig. 4d), although a small increase in *hASMase* mRNA was observed with DA treatment (Fig. 2a). Further experiments are needed to elucidate this issue.

Total transcription activity by Sp family transcription factors after DA-treatment

Using a Sp1 (2) Translucent vector containing two stretches of consensus Sp1 binding motifs, we evaluated the functional change of Sp family transcription factor(s) between control- and DA-treated MCF-7 cells. Our results showed that DA-treated MCF-7 cells were 5 times more potent in activating these consensus Sp1 binding motifs than control MCF-7 cells, while the increase in relative luciferase activity of control vectors was nearly doubled (Fig. 5a). Figure 5b shows Western blotting of Sp1 and Sp3 proteins of MCF-7 cells with or without DA treatment. Interestingly, full-length Sp3 but not Sp1 increased with DA treatment. No remarkable change was observed in the short form (splice variant) of Sp3. Mithramycin A (500 nM), an inhibitor of the Sp family transcription factors, inhibited *hNSMase2* mRNA expression of MCF-7 cells (Fig. 5c). Similarly, the combination of siRNAs of *Sp1* and *Sp3* but not each single siRNA inhibited *NSMase2* mRNA, although either the siRNA of *Sp1* and *Sp3* we used according to previous reports could inhibit *Sp1* and *Sp3* mRNA partially (at most 50%), probably due to the high endogenous expression levels in MCF-7 cells. In DA-treated cells the effects of siRNAs of *Sp1* and *Sp3* were less than those observed in control cells (data not shown), probably due to the increase of Sp3 mRNA as well as the limited inhibition efficiency of these siRNAs as shown in control cells.

In order to evaluate the transcription promoting activity of the Sp family proteins, Sp1 and Sp3, we used a drosophila cell line, SL2, a cell line frequently employed in the analysis of Sp family protein function since it lacks Sp family proteins. Co-transfection of *Sp1* or *Sp3* expression vectors to the drosophila cells with the -148 bp *NSMase2* luc reporter (Fig. 5d) showed that both Sp1 and Sp3 overexpression (inset) induced *NSMase2* promoter activity. Together with the cellular Sp protein level shown in Fig. 5b, these results suggest the importance of Sp family proteins as the major

DA-induced transcription factor of *NSMase2*.

Electrophoresis mobility shift assay (EMSA) and ChIP assay

In Figure 6, EMSA was shown using three Sp1 binding motifs as a probe illustrated in Fig. 4c. Wild (WT) oligo *a* produced four bands, 1, 2, 3 and 4. DA treatment increased band 1 and 2 (6a). Mutated (Mut) oligo (containing a mutated Sp1 motif) did not produce bands, 1 and 2. A cold competitor decreased bands *1* and *2* but not *3* and *4*. Incubation with anti-Sp1 and anti-Sp3 antibody decreased DA-induced band 1 and 2, and produced supershifted bands (shown as ss), respectively, suggesting the presence of Sp1 in the band 1 and Sp3 in the band 2, respectively. Wild (Wt) oligo *b* produced two bands, 1 and 2 (6b). The specificity of these bands was proved with the cold competitor. DA increased band 1 slightly. Anti-Sp1 antibody decreased band 1 intensity. Wild (WT) oligo *c* produced two bands, 1 and 2 (6c). DA treatment increased band 1. Mutated (Mut) oligo (containing a mutated Sp1 motif) did not produce bands, 1 and 2. A cold competitor abolished these bands (c, upper part). Furthermore, mutated cold competitors did not change band 1 pattern (c, lower part). Interestingly, incubation with anti-Sp3 but not anti-Sp1 antibody decreased DA-induced band 1 (c, middle part), suggesting that Sp3 was specially involved with the binding of this *c* motif.

Figure 7 showed the results of ChIP assay, which clearly illustrated that not only Sp3 but also Sp1 was bound to these Sp1 motifs and Sp1 binding was increased with DA, while Sp3 binding was weak and remained at the same level under our experimental conditions.

Discussion

Many lines of evidence have recognized ceramide as a potent proapoptotic mediator [3, 25]. In eukaryotic cells, there are two main pathways to produce ceramide. One is the de novo synthesis pathway starting from serine and palmitoyl CoA, and the other is the SM hydrolysis pathway. In fact, hydrolysis of SM leading to the accumulation of ceramide has been reported in various cell types [26, 27].

NSMase has been identified, characterized and cloned from bacteria, yeast and mammalian cells [28]. Among SMases, Mg^{2+}-dependent NSMase has been thought to play a role in stress-induced ceramide production. However, until only recently, no precise characterization of this enzyme had been demonstrated. Though NSMase1 was reported first, it is less likely to be implicated in the apoptotic process, since overexpression of *NSMase1* affected neither cellular SM nor ceramide contents [6]. A recent report, however, showed some involvement of zebrafish NSMase1 in the process of apoptosis [29].

It should be noted that, overexpression of *NSMase2* or suppression of *NSMase2* by siRNA caused changes in SM metabolites [7]. Interestingly, Hayashi et al. [30] reported that the gene expression of a rat homologue of *NSMase2* was enhanced when cells became confluent, which was consistent with our present study. The importance of *NSMase2* in leukemogenesis (decreased *NSMase2* mRNA level and the presence of mutation) has been shown by several reports including ours [10, 11]. Concerning the effect of an anti-cancer drug, we have recently reported that NSMase2 activity is involved in anti-cancer drug (oxaliplatin)-induced cell death of colon cancer cell lines [31]. Very recently, the involvement of NSMase3 in DNA damage responses was also reported [32], but the temporal changes in *NSMase3* mRNA after treatment with the anti-cancer drug adriamycin was quite different from those of *NSMase2*. *NSMase3* mRNA was transiently increased but decreased thereafter, prompting questions as to its role in prolonged ceramide production during anti-cancer drug treatment.

SMases involving ceramide generation induced by DA has been intensively studied and reviewed [33]. Orgetmen et al. [34] reported a DA-induced increase of cellular ceramide levels. Another study has described a brief period of NSMase activation by DA [35]. Our quantitative RT-PCR

analysis has consistently revealed that DA and cellular confluence induced a long-term gene expression of *NSMase2,* but not of *NSMase1* or *NSMase 3* (Fig. 2). The increase in *ASMase* mRNA was less distinct compared to the one in *NSMase2*. Our data also showed DA-induced increases in C16, C18 and C24 ceramide, which is consistent with the data by Marchesini et al. [8]. Increases in cellular ceramide (Fig. 1a) and NSMase2 protein levels by DA (Fig. 2c) were well correlated with the gradual increase in the *NSMase2* message level (Fig. 2b). The involvement of NSMase2 in the DA sensitivity of MCF-7 cells was shown by the overexpression and knock down experiments of *NSMase2* (Fig. 3). Our results were consistent with the effect of a chemical NSMase 2 inhibitor, GW4869, on another anti-cancer drug oxaliplatin-induced cell death reported recently [31]. It is important to know how much NSMase 2 is involved in total NSMase enzyme activity. It might help elucidate the mechanism of sustained SMase activation during cancer chemotherapy. However, we could not directly explore this issue because of the absence of suitable NSMase 1 and NSMase 3 antibodies available commercially. In the preliminary experiments, we observed the decrease of NSMase enzyme activity after immunodepletion of NSMase2 protein. However, further analysis is needed to determine the respective contribution of each NSMase isoform in the total NSMase enzyme activity. siRNA of *NSMase2* reported by others [8] induced a fair suppression of mRNA and protein in a small-scale of culture as shown in Fig. 2. However, when we examined the effect of siRNA of NSMase2 on enzyme activity in a large-scale culture to obtain sufficient samples needed for the enzyme assay, heterogeneous levels of inhibition among experiments were observed, and we could not determine the exact percentage of NSMase2 contribution to the DA-induced increase of total NSMase enzyme activity (data not shown). Further analysis is needed to enhance the inhibition by *NSMase2* siRNA in a large-scale culture. Therefore, our present results suggested but did not prove completely that NSMase2 might be the main NSMase isoform in ceramide production in the DA-induced apoptotic process.

Transcriptional regulation of *SMase* is one of the important mechanisms of sustained SMase enzyme activity. The promoter analysis of *ASMase* was reported previously [13, 36]. However, the 5' promoters of *NSMase2* and *NSMase1* have not been analyzed adequately. Our current analysis clearly showed that basal and DA-induced *NSMase2* promoter activity was located

between –148 bp and –42 bp of 5' promoter of *NSMase2*, whereas DA did not induce either *ASMase* or *NSMase1* promoter activities significantly (Fig. 4). Another analysis revealed that DA up-regulated expression of several genes whose promoters have Sp1 motif [37]. Actually, Sp1 transcription factor has been implicated in the gene expression of DA-induced *glucosylceramide synthase*, which is another important enzyme for regulating cellular ceramide level [38, 39].

We found that the region between -148 bp and -42 bp of the 5' promoter where three Sp1 motifs located was important for its promoter activity. Among the three Sp1 motifs (*a, b* and *c*), the proximal *c* motif was the most potent (Fig. 4c line c). However, the introduction of a mutation into two other Sp1 motifs also decreased *NSMase2* promoter activity; the combination of three intact Sp1 motifs showed higher promoter activity compared to a single intact Sp1 motif, suggesting the synergistic action of all three Sp1 motifs. The effect of mithramycin A on *NSMase2* mRNA levels supports the involvement of Sp family proteins in *NSMase2* gene transcription (Fig. 5c). The combination of *Sp1* and *Sp3* siRNAs could also inhibit *NSMase2* mRNA, although the inhibition is at most 50%, probably due to the high endogenous Sp family gene expression. This is because a single siRNA of *Sp1* or *Sp3* did not inhibit *NSMase2* mRNA, suggesting the cooperative role of Sp1 and Sp3 in *NSMase2* gene expression. Using SL2 cells lacking Sp family transcription factors (Fig. 5d), both Sp1 and Sp3 have the potency to induce *NSMase2* promoter activity.

Sp1 is generally known to activate transcription, while Sp3 is a dual-role transcription factor that acts as either an activator or a repressor depending on the promoter context [40-42]. The DNA binding domains of Sp1 and Sp3 are essentially identical in size, conformation and DNA recognition [43, 44]. Sp1 protein has been reported to play the major role in *TRAIL* and *glucosylcermide synthase* transcription in adriamycin- or doxorubicin-treated cell lines [38, 45]. In MCF-7 cells, the promoter analysis using Sp1 translucent vector suggests the increased activity of Sp family transcription factor, and Sp3 but not Sp1 protein level increased after DA treatment (Fig. 5a, b). Increase of cellular Sp3 suggests the direct role of Sp3 in DA-induced increase in NSMase2 transcription. These results suggest that NSMase2 induced by anti-cancer drugs, DA, was controlled by various (or combinations of) Sp family proteins.

Next, we examined all three Sp1 motifs by EMSA (Fig. 6). The results clearly show the complex binding of Sp proteins and 3 Sp motifs with DA treatment. In motif *a* (left side), DA increased the intensity of bands 1 and *2,* which contained both Sp1 and Sp3 confirmed by the supershift assay. According to these results, band 1 contains Sp1, whereas band 2 contains Sp3. In motif b, increased band 1 with DA treatment was suppressed by anti-Sp1. Interestingly, in motif *c* (right), EMSA showed DA-induced increase of specific band *1.* Supershift assay using anti-Sp3 antibody revealed the involvement of Sp3 but not Sp1 protein binding. These results show the complex binding mode of Sp family proteins to these Sp1 motifs of NSMase2 promoter of DA-treated MCF-7 cells, and support our reporter assay shown in Fig. 4. In the ChIP assay, we also confirmed binding of Sp1 and Sp3 to these three Sp1 motifs (Fig. 7a).

Taken together, the present study showed for the first time that *NSMase2* mRNA displayed the most remarkable increase compared with other SMases when treated with DA, and that this sustained increase of *NSMase2* mRNA was correlated with ceramide production leading to cancer cell death. Our results also suggest that the ubiquitous transcription factor, Sp family, is involved with *NSMase2* transcription and that DA increased binding of Sp family proteins and cis-elements of 5' promoter of *NSMase2* gene in a complexed way.

References

[1] S. Spiegel, A.H. Merrill, Jr., Sphingolipid metabolism and cell growth regulation, FASEB J 10 (1996) 1388-1397.

[2] Y.A. Hannun, L.M. Obeid, The Ceramide-centric universe of lipid-mediated cell regulation: stress encounters of the lipid kind, J. Biol. Chem. 277 (2002) 25847-25850.

[3] G.S. Dbaibo, Y.A. Hannun, Signal transduction and the regulation of apoptosis: roles of ceramide, Apoptosis 3 (1998) 317-334.

[4] V. Mansat, G. Laurent, T. Levade, A. Bettaieb, J.P. Jaffrezou, The protein kinase C activators phorbol esters and phosphatidylserine inhibit neutral sphingomyelinase activation, ceramide generation, and apoptosis triggered by daunorubicin, Cancer Res. 57 (1997) 5300-5304.

[5] N. Marchesini, Y.A. Hannun, Acid and neutral sphingomyelinases: roles and mechanisms of regulation, Biochem Cell Biol 82 (2004) 27-44.

[6] H. Sawai, N. Domae, N. Nagan, Y.A. Hannun, Function of the cloned putative neutral sphingomyelinase as lyso-platelet activating factor-phospholipase C, J. Biol. Chem. 274 (1999) 38131-38139.

[7] N. Marchesini, C. Luberto, Y.A. Hannun, Biochemical properties of mammalian neutral sphingomyelinase 2 and its role in sphingolipid metabolism, J. Biol. Chem. 278 (2003) 13775-13783.

[8] N. Marchesini, W. Osta, J. Bielawski, C. Luberto, L.M. Obeid, Y.A. Hannun, Role for mammalian neutral sphingomyelinase 2 in confluence-induced growth arrest of MCF7 cells, J. Biol. Chem. 279 (2004) 25101-25111.

[9] W. Stoffel, B. Jenke, B. Block, M. Zumbansen, J. Koebke, Neutral sphingomyelinase 2 (smpd3) in the control of postnatal growth and development, Proc. Natl. Acad. Sci. USA 102 (2005) 4554-4559.

[10] S. Sobue, T. Iwasaki, C. Sugisaki, K. Nagata, R. Kikuchi, M. Murakami, A. Takagi, T. Kojima, Y. Banno, Y. Akao, Y. Nozawa, R. Kannagi, M. Suzuki, A. Abe, T. Naoe, T. Murate, Quantitative RT-PCR analysis of sphingolipid metabolic enzymes in acute leukemia and myelodysplastic syndromes, Leukemia 20 (2006) 2042-2046.

[11] W.J. Kim, R.A. Okimoto, L.E. Purton, M. Goodwin, S.M. Haserlat, F. Dayyani, D.A. Sweetser, A.I. McClatchey, O.A. Bernard, A.T. Look, D.W. Bell, D.T. Scadden, D.A. Haber, Mutations in the neutral sphingomyelinase gene SMPD3 implicate the ceramide pathway in human leukemias, Blood 111 (2008) 4716-4722.

[12] O. Krut, K. Wiegmann, H. Kashkar, B. Yazdanpanah, M. Kronke, Novel tumor

necrosis factor-responsive mammalian neutral sphingomyelinase-3 is a C-tail-anchored protein, J. Biol. Chem. 281 (2006) 13784-13793.

[13] T. Murate, M. Suzuki, M. Hattori, A. Takagi, T. Kojima, T. Tanizawa, H. Asano, T. Hotta, H. Saito, S. Yoshida, K. Tamiya-Koizumi, Up-regulation of acid sphingomyelinase during retinoic acid-induced myeloid differentiation of NB4, a human acute promyelocytic leukemia cell line, J. Biol. Chem. 277 (2002) 9936-9943.

[14] F. Liu, N. Pore, M. Kim, K.R. Voong, M. Dowling, A. Maity, G.D. Kao, Regulation of histone deacetylase 4 expression by the SP family of transcription factors, Mol Biol Cell 17 (2006) 585-597.

[15] K. Yamada, T. Tanaka, K. Miyamoto, T. Noguchi, Sp family members and nuclear factor-Y cooperatively stimulate transcription from the rat pyruvate kinase M gene distal promoter region via their direct interactions, J. Biol. Chem. 275 (2000) 18129-18137.

[16] S. Satoh, S. Masatoshi, Z. Shou, T. Yamamoto, T. Ishigure, A. Semii, K. Yamada, T. Noguchi, Identification of cis-regulatory elements and trans-acting proteins of the rat carbohydrate response element binding protein gene, Arch. Biochem. Biophys. 461 (2007) 113-122.

[17] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can J Biochem Physiol 37 (1959) 911-917.

[18] S. Sobue, S. Nemoto, M. Murakami, H. Ito, A. Kimura, S. Gao, A. Furuhata, A. Takagi, T. Kojima, M. Nakamura, Y. Ito, M. Suzuki, Y. Banno, Y. Nozawa, T. Murate, Implications of sphingosine kinase 1 expression level for the cellular sphingolipid rheostat: relevance as a marker for daunorubicin sensitivity of leukemia cells, Int J Hematol 87 (2008) 266-275.

[19] M.M. Ling, B.H. Robinson, Approaches to DNA mutagenesis: an overview, Anal Biochem 254 (1997) 157-178.

[20] S. Sobue, K. Hagiwara, Y. Banno, K. Tamiya-Koizumi, M. Suzuki, A. Takagi, T. Kojima, H. Asano, Y. Nozawa, T. Murate, Transcription factor specificity protein 1 (Sp1) is the main regulator of nerve growth factor-induced sphingosine kinase 1 gene expression of the rat pheochromocytoma cell line, PC12, J Neurochem 95 (2005) 940-949.

[21] S. Sobue, M. Murakami, Y. Banno, H. Ito, A. Kimura, S. Gao, A. Furuhata, A. Takagi, T. Kojima, M. Suzuki, Y. Nozawa, T. Murate, v-Src oncogene product increases sphingosine kinase 1 expression through mRNA stabilization: alteration of AU-rich element-binding proteins, Oncogene (2008).

[22] S. Li, Y. Zhou, Y. Dong, C. Ip, Doxorubicin and selenium cooperatively induce fas

signaling in the absence of Fas/Fas ligand interaction, Anticancer Res. 27 (2007) 3075-3082.

[23] S. Wang, E.A. Konorev, S. Kotamraju, J. Joseph, S. Kalivendi, B. Kalyanaraman, Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. intermediacy of H(2)O(2)- and p53-dependent pathways, J. Biol. Chem. 279 (2004) 25535-25543.

[24] S. Grazide, N. Maestre, R.J. Veldman, C. Bezombes, S. Maddens, T. Levade, G. Laurent, J.P. Jaffrezou, Ara-C- and daunorubicin-induced recruitment of Lyn in sphingomyelinase-enriched membrane rafts, FASEB J 16 (2002) 1685-1687.

[25] M. Schenck, A. Carpinteiro, H. Grassme, F. Lang, E. Gulbins, Ceramide: physiological and pathophysiological aspects, Arch. Biochem. Biophys. 462 (2007) 171-175.

[26] N. Andrieu-Abadie, T. Levade, Sphingomyelin hydrolysis during apoptosis, Biochim. Biophys. Acta 1585 (2002) 126-134.

[27] L. Riboni, P. Viani, R. Bassi, A. Prinetti, G. Tettamanti, The role of sphingolipids in the process of signal transduction, Prog Lipid Res 36 (1997) 153-195.

[28] C.J. Clarke, C.F. Snook, M. Tani, N. Matmati, N. Marchesini, Y.A. Hannun, The extended family of neutral sphingomyelinases, Biochemistry 45 (2006) 11247-11256.

[29] T. Yabu, S. Imamura, M. Yamashita, T. Okazaki, Identification of Mg2+-dependent neutral sphingomyelinase 1 as a mediator of heat stress-induced ceramide generation and apoptosis, J. Biol. Chem. (2008).

[30] Y. Hayashi, T. Kiyono, M. Fujita, M. Ishibashi, cca1 is required for formation of growth-arrested confluent monolayer of rat 3Y1 cells, J. Biol. Chem. 272 (1997) 18082-18086.

[31] S. Nemoto, M. Nakamura, Y. Osawa, S. Kono, Y. Itoh, Y. Okano, T. Murate, A. Hara, H. Ueda, Y. Nozawa, Y. Banno, Sphingosine kinase isoforms regulate oxaliplatin sensitivity of human colon cancer cells through ceramide accumulation and AKT activtion, J. Biol. Chem. (2009).

[32] C.A. Corcoran, Q. He, S. Ponnusamy, B. Ogretmen, Y. Huang, M.S. Sheikh, Neutral sphingomyelinase-3 is a DNA damage and nongenotoxic stress-regulated gene that is deregulated in human malignancies, Mol. Cancer Res. 6 (2008) 795-807.

[33] G. Laurent, J.P. Jaffrezou, Signaling pathways activated by daunorubicin, Blood 98 (2001) 913-924.

[34] B. Ogretmen, J.M. Kraveka, D. Schady, J. Usta, Y.A. Hannun, L.M. Obeid, Molecular mechanisms of ceramide-mediated telomerase inhibition in the A549 human lung adenocarcinoma cell line, J. Biol. Chem. 276 (2001) 32506-32514.

[35] J.P. Jaffrezou, T. Levade, A. Bettaieb, N. Andrieu, C. Bezombes, N. Maestre, S. Vermeersch, A. Rousse, G. Laurent, Daunorubicin-induced apoptosis: triggering of ceramide generation through sphingomyelin hydrolysis, EMBO J. 15 (1996) 2417-2424.

[36] T. Langmann, C. Buechler, S. Ries, A. Schaeffler, C. Aslanidis, M. Schuierer, M. Weiler, K. Sandhoff, P.J. de Jong, G. Schmitz, Transcription factors Sp1 and AP-2 mediate induction of acid sphingomyelinase during monocytic differentiation, J. Lipid Res. 40 (1999) 870-880.

[37] S. Mansilla, J. Portugal, Sp1 transcription factor as a target for anthracyclines: effects on gene transcription, Biochimie 90 (2008) 976-987.

[38] Y.Y. Liu, J.Y. Yu, D. Yin, G.A. Patwardhan, V. Gupta, Y. Hirabayashi, W.M. Holleran, A.E. Giuliano, S.M. Jazwinski, V. Gouaze-Andersson, D.P. Consoli, M.C. Cabot, A role for ceramide in driving cancer cell resistance to doxorubicin, FASEB J 22 (2008) 2541-2551.

[39] Y. Uchida, M. Itoh, Y. Taguchi, S. Yamaoka, H. Umehara, S. Ichikawa, Y. Hirabayashi, W.M. Holleran, T. Okazaki, Ceramide reduction and transcriptional up-regulation of glucosylceramide synthase through doxorubicin-activated Sp1 in drug-resistant HL-60/ADR cells, Cancer Res. 64 (2004) 6271-6279.

[40] G. Hagen, S. Muller, M. Beato, G. Suske, Sp1-mediated transcriptional activation is repressed by Sp3, EMBO J. 13 (1994) 3843-3851.

[41] B. Majello, P. De Luca, G. Hagen, G. Suske, L. Lania, Different members of the Sp1 multigene family exert opposite transcriptional regulation of the long terminal repeat of HIV-1, Nucleic Acids Res. 22 (1994) 4914-4921.

[42] B. Yu, P.K. Datta, S. Bagchi, Stability of the Sp3-DNA complex is promoter-specific: Sp3 efficiently competes with Sp1 for binding to promoters containing multiple Sp-sites, Nucleic Acids Res. 31 (2003) 5368-5376.

[43] P. Bouwman, S. Philipsen, Regulation of the activity of Sp1-related transcription factors, Mol Cell Endocrinol. 195 (2002) 27-38.

[44] G. Suske, The Sp-family of transcription factors, Gene 238 (1999) 291-300.

[45] J. Xu, J.Y. Zhou, W.Z. Wei, S. Philipsen, G.S. Wu, Sp1-mediated TRAIL induction in chemosensitization, Cancer Res. 68 (2008) 6718-6726.

Legend to Figures

Figure 1. DA-induced changes of cellular ceramide levels and SMase activity.

(a) Cellular caspase 3 activities were analyzed as indicated in the Materials and methods. Cells were cultured in triplicate with DA (0.5 μM) for 48 h. The data were expressed as the mean +/- SD. The activity of control cells on day 0 was regarded as 1.0.

(b) Cells were treated and collected as described above. Viable cells and apoptotic cells were counted according to the Materials and methods. Viable cell number of Day 0 control cells and cells treated with DA for 48 h were regarded as 100%. Apoptotic cells were expressed as the percentage of at least 300 cells surveyed.

(c) MCF-7 cells were treated with or without 0.5 μM of DA for 24 h. Cellular ceramide levels were measured by LC/MS-MS method as described in Materials and methods. Assay was performed in triplicate and the result was shown from three independent experiments. In each ceramide molecule, the quantity of cells without DA-treatment was regarded as 1.0.

(d) NSMase and ASMase enzyme activities were measured in MCF-7 cells with or without DA-treatment for 24 h as described in Materials and methods. Assay was performed in triplicate and the mean +/- SD was established from three independent experiments. Enzyme activity of cells without DA treatment was regarded as 1.0. NSMase activity of control MCF7 cells was 7.37 +/- 0.38 nmol hydrolyzed SM/mg protein/30 min, whereas that of ASMase activity was 10.49 +/- 0.28.

Figure 2. Measurement of *hSMase* mRNAs and NSMase 2 protein level

(a) *hNSMase1, 2* and *3* and *ASMase* mRNA levels were measured in MCF-7 cells treated with 0.5 μM of DA for 24 h or MCF-7 cells that reached their confluent state at 48 h. mRNA levels were measured by the real-time RT-PCR method as described in Materials and methods. The mRNA level of control MCF-7 cells was regarded as 1.0. Mean +/- SD was demonstrated by three independent experiments.

(b) Temporal changes of *hNSMase2* message level were measured at various times after 0.5 μM of DA treatment. Solid diamond denotes MCF-7 cells

without DA treatment, whereas solid square shows cells treated with DA.
(c) Western blotting of NSMase2 was performed as described in Materials and methods. DA denotes MCF7 cells treated with DA for 24 h. Confluent cells were collected 48 h after reaching their confluent state.

Figure 3. Effect of modulating *NSMase 2* expression on DA-sensitivity of MCF-7 cells.

(a) MCF-7 cells stably overexpressing FLAG-tagged *mNSMase2* were established and subjected to Western blotting using anti-FLAG antibody. Mock-MCF-7 cells were shown as the control. NSMase enzyme activity of each transfectant was measured as described in Materials and methods. Activity of mock-MCF-7 cells without DA treatment was determined as 1.0
(b) Using these two stable subclones (mock- and mNSMase2-MCF-7), DA sensitivity was examined by counting viable cell numbers after DA treatment (0.5 μM).
Mean +/- SD was shown. Mock transfectant without DA treatment was regarded as 1.0. Asterisk denotes statistical significance between the two groups ($p< 0.05$)
(c) Either scrambled or *hNSMase2* siRNA was transfected into MCF-7 cells as described in Materials and methods. *hNSMase2* mRNA level was measured by quantitative RT-PCR. NSMase2 protein level was also shown in inset.
(d) After each siRNA was transfected, cells were treated with 0.5 μM of DA, and remaining viable cell numbers were counted in triplicate. Scrambled siRNA-treated cells without DA treatment were regarded as 1.0. Asterisk denotes statistical significance between the two groups ($p< 0.05$).

Figure 4. Promoter analysis of *hNSMase2*, *hNSMase1* and *hASMase* genes.

(a) and (b) 5' region of *hNSMase2* promoter was examined for promoter activity using various truncated luciferase vectors as shown on the left side of the Figure. The transcription start site was determined as +1. Assay was performed in triplicate and experiments were repeated at least three times. The result of -42 bp/+109 bp luc without DA treatment was regarded as 1.0. The localization of Sp1 sites was illustrated in (b).
(c) Luciferase vectors containing a mutated Sp1 site were also prepared as

described in Materials and methods, and results were shown. An open diamond shows a wild Sp1 site, while a solid one denotes a mutated Sp1 site. (d) Similarly, the promoter analysis of *hNSMase1* and *hASMase* was presented. The result without DA treatment, DA (-), was regarded as 1.0. The preparation of *hNSMase1* luciferase vector (-938 bp/+81 bp Luc) was described in Materials and methods. *hASMase* luciferase vector (-519 bp/+300 bp Luc) was reported previously [13].

Figure 5. Effects of Sp family transcription factors in DA-induced *hNSMase2* gene expression.

(a) MCF-7 cells were transfected in triplicate with either control Translucent vector or

Sp1 (2) Translucent reported vector (3 μg each) together with 2 μg of β-gal expression vector. Relative activity was presented as Luc/β-gal. The mean +/- SD was shown. Data of control vector-transfected cells without DA treatment were regarded as 1.0.

(b) Cellular Sp1 and Sp3 levels were analyzed by Western blotting using anti-Sp1 or anti-Sp3 antibody as described in Materials and methods. β-actin was shown as the internal control. M1 and M2 denote splice variants of Sp3 protein.

(c) Effect of mithramycin A (MMA), an inhibitor of Sp family protein, on *NSMase2* mRNA. MCF-7 cells treated with or without MMA (500 nM) for 12 h were collected and their *hNSMase2* mRNA levels were measured by semi-quantitative RT-PCR. Similarly, siRNA of *Sp1*, siRNA of *Sp3*, or both were transfected to MCF-7 cells for 48 h, and *NSmase2* mRNA was measured. *NSMase2* mRNA levels were expressed as *NSMase2/GAPDH*. Cycle denotes the number of PCR cycles.

(d) pPac-Sp1, pPac-USp3 (full length) expression vectors for drosophila SL2 cells or control pPac were transfected transiently to SL2 cells in triplicate together with pPac-RL and -148 bp/+109 bp *NSMase2* luc vector. pPac RL was used to correct transfection efficiency. Inset shows Western blotting result of each Sp protein with its respective expression vector. Luciferase activity was calculated as the relative values to -148 bp/+108 bp *NSMase2* luc/pPac-RL. Samples transfected with (control) pPac vector were regarded as 1.0. The mean +/- SD was shown.

Figure 6. EMSA.

Using 3 labeled oligoprobes (*a*, *b*, and *c*) as shown in Table 2, EMSA was performed. Italic letters in Table 2 denote mutated Sp1 motif.

(a) The results of probe *a* were shown. Both wild-type and mutated-type (Mut)-labeled oligonucleotides were used. DA denotes DA treatment for 24 h. Cold means cold competitor addition. Supershift experiments using anti-Sp1 and anti-Sp3 antibodies were illustrated below. ss means a supershift band.

(b) (c) EMSA using labeled probe *b* and *c* were shown. DA(+) and (-) show DA-treated and control MCF-7 cells, respectively. Cold means cold competitor addition. Mut denotes mutated labeled probe, whereas Mut cold is the cold mutated competitor addition. Cold competitor (x4 and x64) was added in competition experiments. In supershift experiments, anti-Sp1 or anti-Sp3 antibody (2 μg/sample) was added to nuclear extracts before incubating with labeled oligonucleotide as described in Materials and methods.

Figure 7. ChIP assay.

(a) ChIP assay was performed using MCF-7 cells with or without DA-treatment as described in Materials and methods. DA(+) and (-) show DA-treated and control MCF-7 cells. Antibodies used were 200 ng/sample. Control IgG represents normal rabbit IgG. We used $3x10^5$ cells for ChIP assay with anti-Sp1 antibody, whereas $1x10^6$ cells were used for ChIP assay with anti-Sp3 antibody. Input PCR band was equivalent to 0.5% of the original cell lysate. PCR bands of anti-Sp1 and anti-Sp3 antibody-treated samples in the figure were equivalent to 6% and 15% of the original cell lysates, respectively.

Fig.1

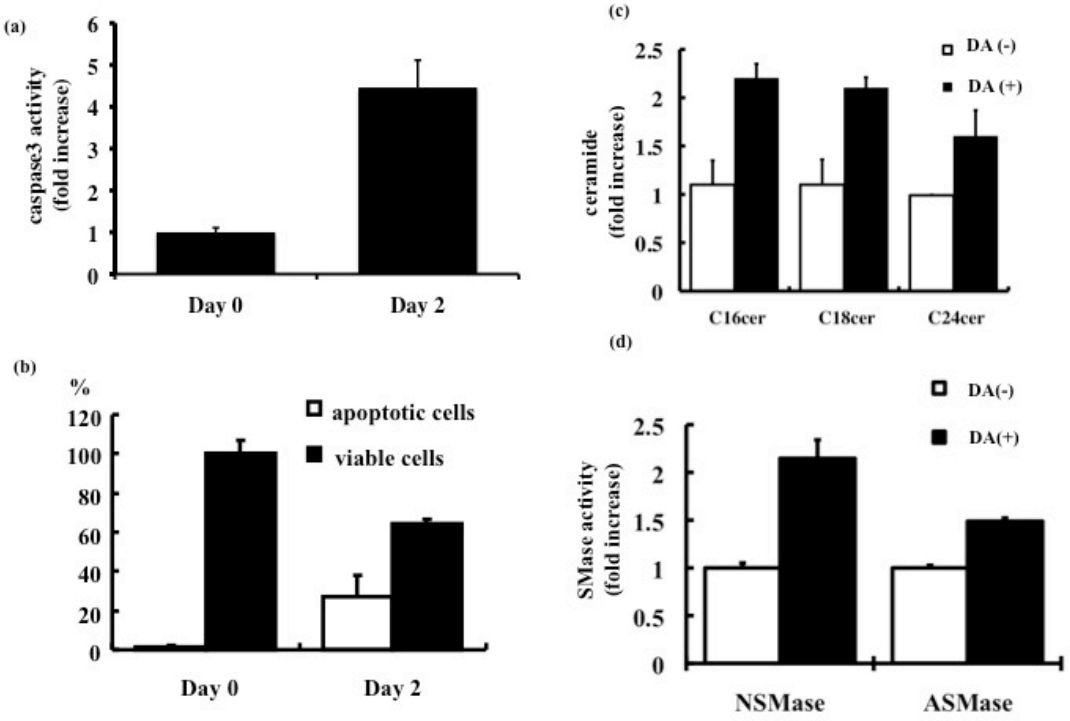

(a)
caspase3 activity (fold increase)
Day 0
Day 2
(b)
%
apoptotic cells
viable cells
Day 0
Day 2
(c)
DA (-)
DA (+)
ceramide (fold increase)
C16cer
C18cer
C24cer
(d)
DA(-)
DA(+)
SMase activity (fold increase)
NSMase
ASMase

Fig.2

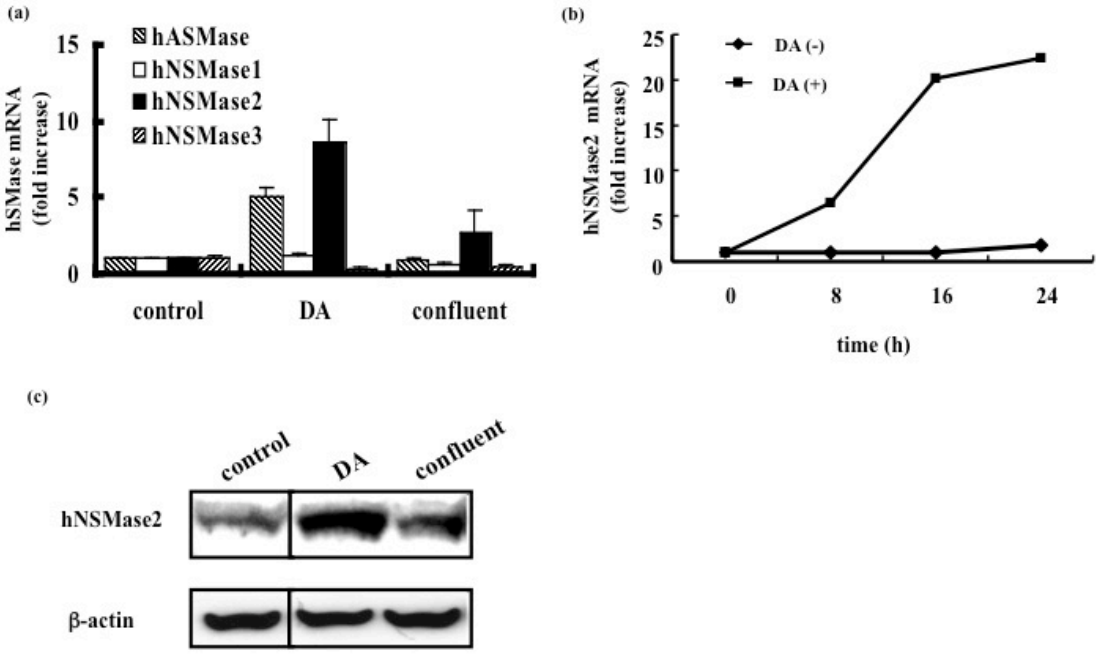

(a)
hASMase
hNSMase1
hNSMase2
hNSMase3
hSMase mRNA (fold increase)
control
DA
confluent
(b)
DA (-)
DA (+)
hNSMase2 mRNA (fold increase)
time (h)
(c)
control
DA
confluent
hNSMase2
β-actin

Fig.3

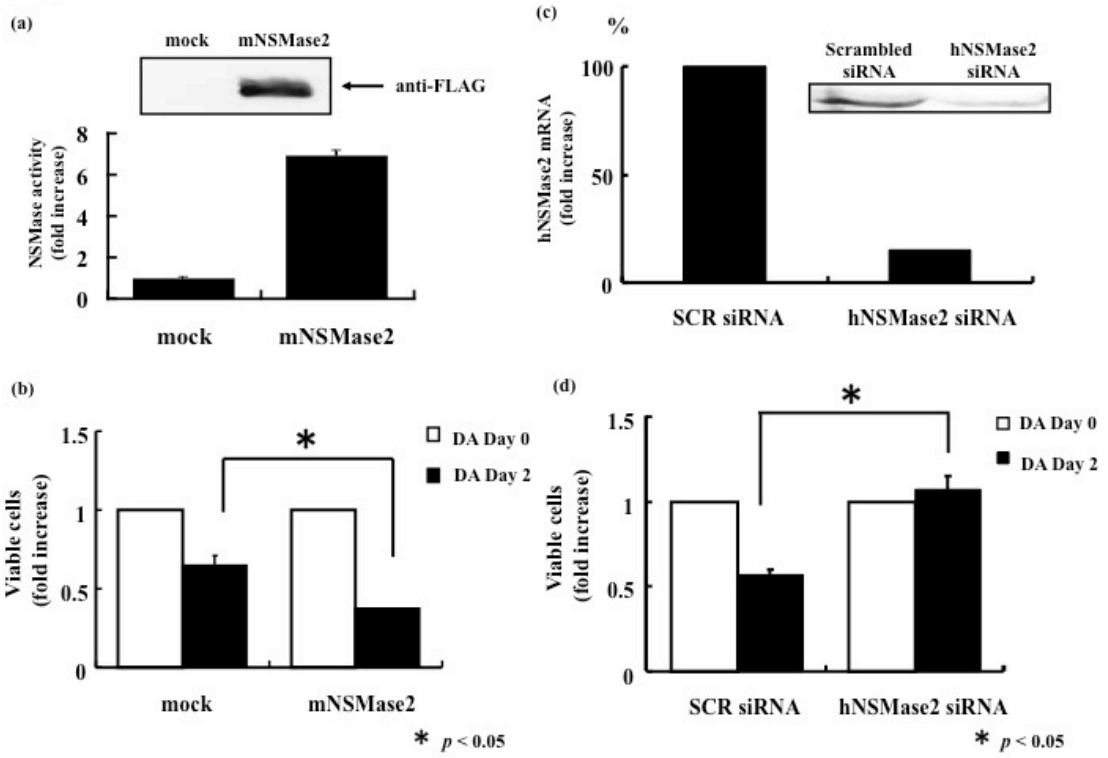

(a)
mock
mNSMase2
anti-FLAG
NSMase activity (fold increase)
8
6
4
2
0
mock
mNSMase2
(c)
%
Scrambled siRNA
hNSMase2 siRNA
hNSMase2 mRNA (fold increase)
100
50
0
SCR siRNA
hNSMase2 siRNA
(b)
Viable cells (fold increase)
1.5
1
0.5
0
*
DA Day 0
DA Day 2
mock
mNSMase2
* p < 0.05
(d)
Viable cells (fold increase)
1.5
1
0.5
0
*
DA Day 0
DA Day 2
SCR siRNA
hNSMase2 siRNA
* p < 0.05

Fig.4

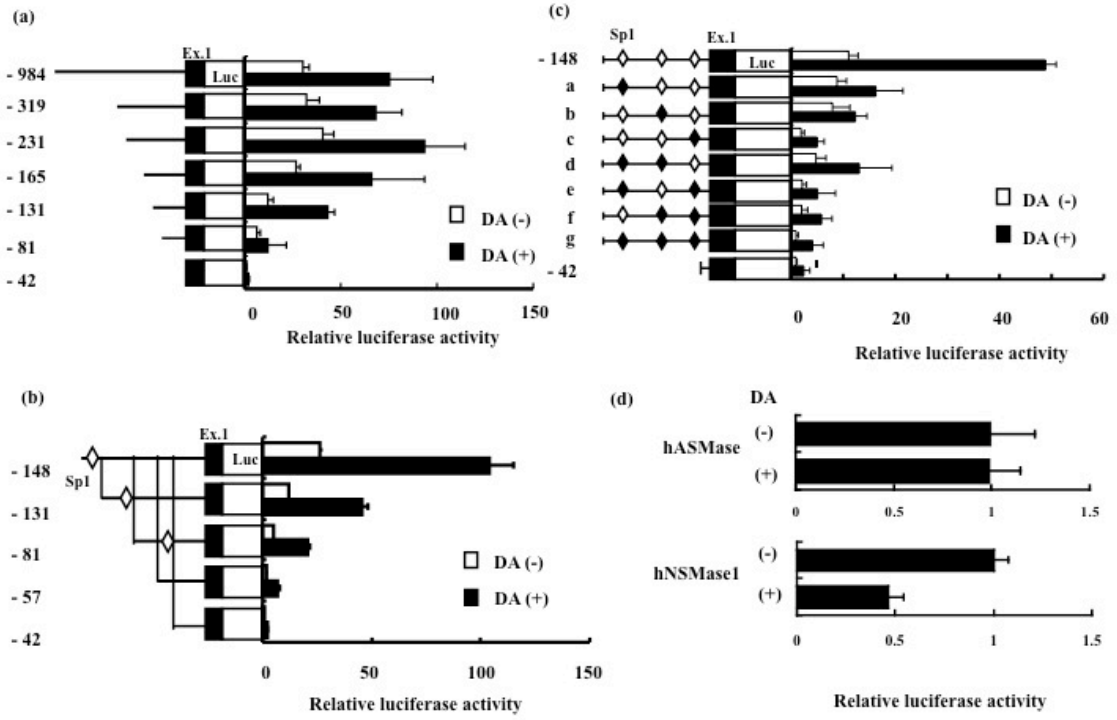

(a)
Ex.1
Luc
- 984
- 319
- 231
- 165
- 131
- 81
- 42
DA (-)
DA (+)
0
50
100
150
Relative luciferase activity
(c)
Sp1
Ex.1
Luc
- 148
a
b
c
d
e
f
g
- 42
DA (-)
DA (+)
0
20
40
60
Relative luciferase activity
(b)
Ex.1
Luc
Sp1
- 148
- 131
- 81
- 57
- 42
DA (-)
DA (+)
0
50
100
150
Relative luciferase activity
(d)
DA
hASMase
(-)
(+)
0
0.5
1
1.5
hNSMase1
(-)
(+)
0
0.5
1
1.5
Relative luciferase activity

Fig. 5

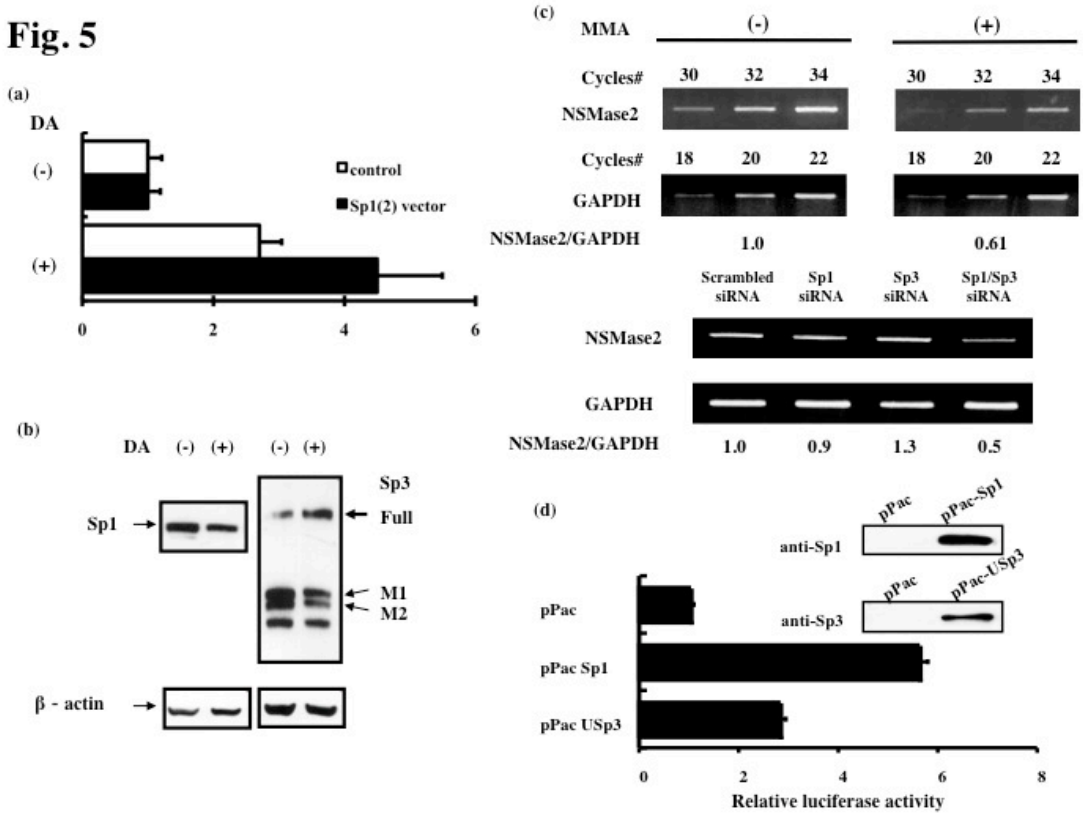

(a)
DA
(-)
(+)
control
Sp1(2) vector
0
2
4
6
(b)
DA (-) (+) (-) (+)
Sp1
Sp3
Full
M1
M2
β - actin
(c)
MMA
(-)
(+)
Cycles#
30 32 34
30 32 34
NSMase2
Cycles#
18 20 22
18 20 22
GAPDH
NSMase2/GAPDH
1.0
0.61
Scrambled siRNA
Sp1 siRNA
Sp3 siRNA
Sp1/Sp3 siRNA
NSMase2
GAPDH
NSMase2/GAPDH
1.0 0.9 1.3 0.5
(d)
pPac
pPac-Sp1
anti-Sp1
pPac
pPac-USp3
anti-Sp3
pPac
pPac Sp1
pPac USp3
0
2
4
6
8
Relative luciferase activity

Fig.6

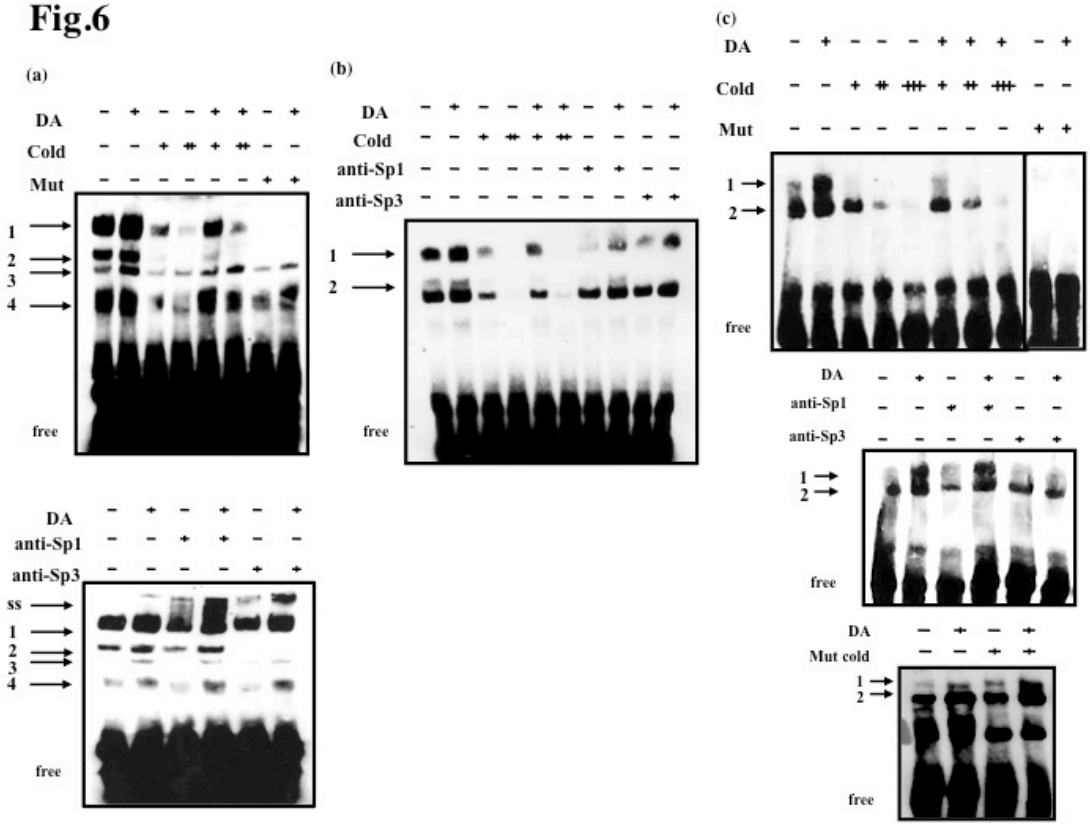

(a)
DA
Cold
Mut
1
2
3
4
free
DA
anti-Sp1
anti-Sp3
ss
1
2
3
4
free
(b)
DA
Cold
anti-Sp1
anti-Sp3
1
2
free
(c)
DA
Cold
Mut
1
2
free
DA
anti-Sp1
anti-Sp3
1
2
free
DA
Mut cold
1
2
free

Fig.7

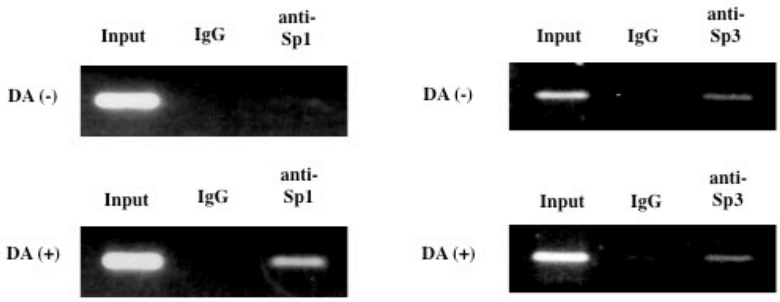


Table 1

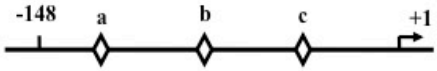
Primer sets of Real time RT-PCR

gene name (accession number)	primer sequence (Forward, Reverse)	annealing temperature
hASMase (NM_000543)	5'-AAGCCCTGCGCACCCTCAGAA-3', 5'-CCTGAAGCTCCCCCACCAGCC-3'	64 ℃
hNSMase1 (NM_003080)	5'-CAGTTCATCCACCACACATCCA-3', 5'-TTGCCTTCCTCAGAGCCCTT-3'	62 ℃
hNSMase2 (NM_018667)	5'-ACTTTGATAACTGCTCCTCTGAC-3', 5'-TTCGTGTCCAGCAGAGTACC-3 '	63 ℃
hNSMase3 (NM_017951)	5'-CCTCATCACTCAGAAGCCA-3', 5'-GAGGCCATAGGAAGCAAA-3'	60 ℃
hGAPDH (NM_002046)	5'-CAGGAGCGAGATCCCTCCAA-3', 5'-CCCCCTGCAAATGAGCCC-3'	63 ℃
h β -actin (NM_001101)	5'-CCGCGAGAAGATGACCCAGA -3', 5'-GTCACCGGAGTCCATCACGA -3'	60 ℃

Primer sets of Semi quantitative RT-PCR

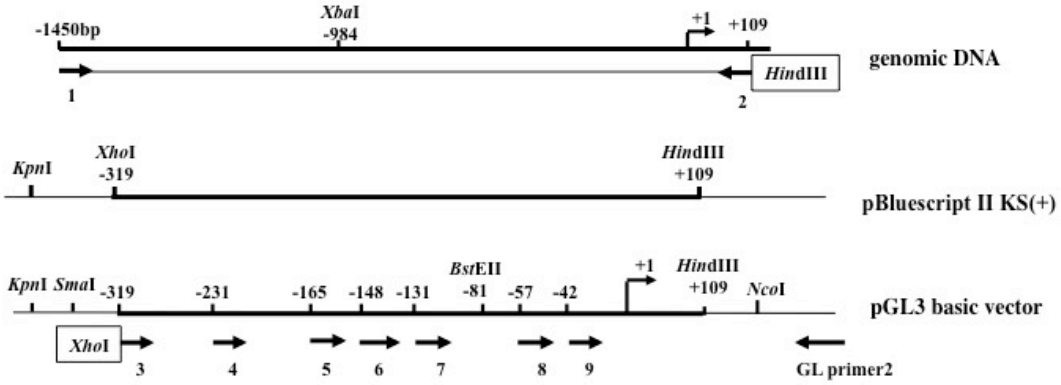
gene name (accession number)	primer sequence (Forward, Reverse)	annealing temperature
hNSMase2 (NM_018667)	5'-TCCTCTTTGCCAGCCGCTAC-3', 5'-CTGCGGCCCTCCTCACTCTC-3'	64 ℃
hGAPDH (NM_ 002046)	5'-GTCAGCCGCATCTTCTTTTG-3', 5'-CGCCAGCATCGCCCCACTTG-3'	61 ℃

Table 2

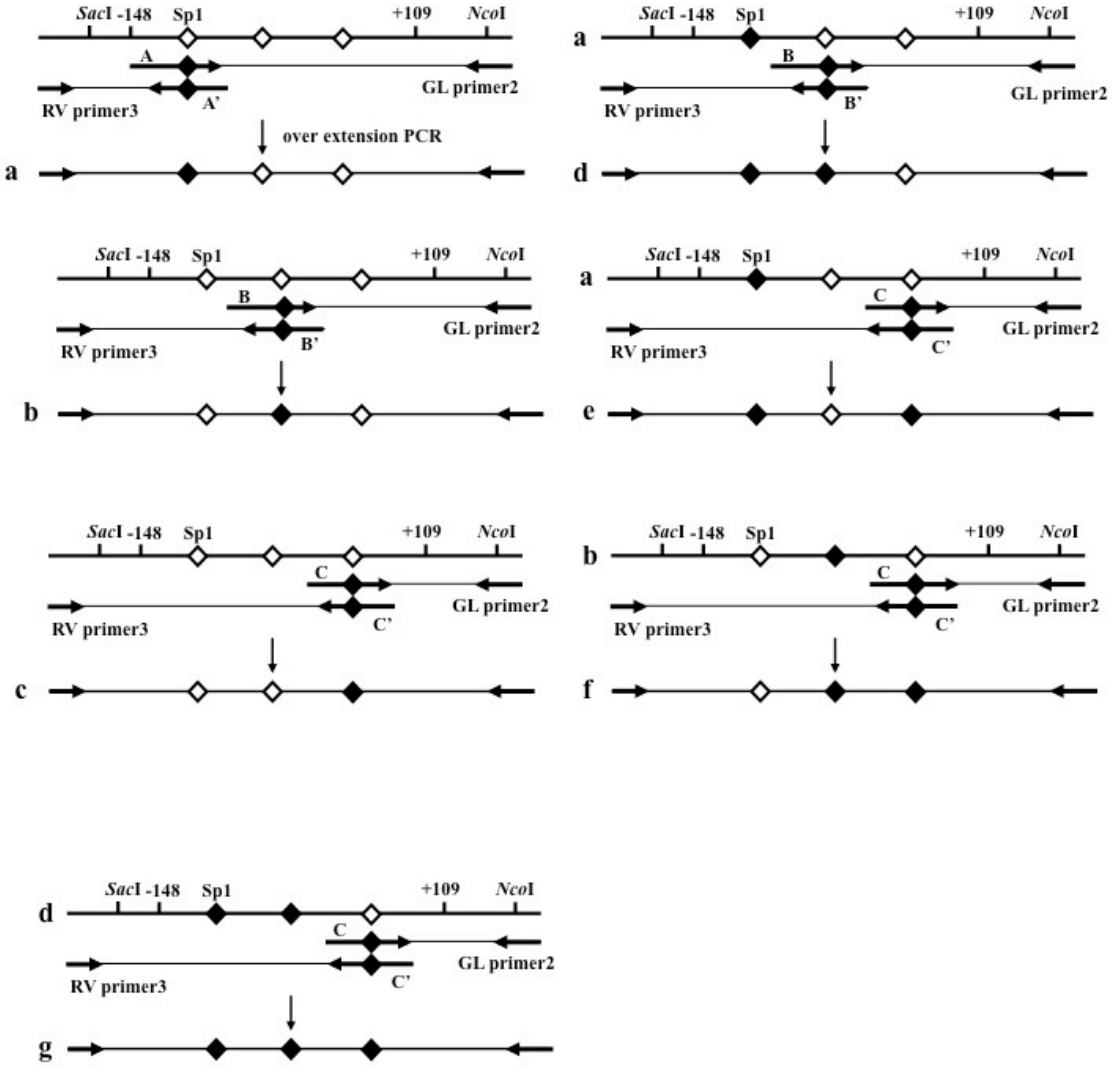


probe a WT	GCAGGAACCTGCGGGCGGGGCCGGAGGGAG
probe a Mut	GCAGGAACCTGC*AGT*CGGGGCCGGAGGGAG
probe b WT	AATGGGCAGACTCCTGGGCGGGGTCCGGGT
probe c WT	CGGCGCGAGGGAGGCGGGGTCAGAGGAATG
probe c Mut	CGGCGCGAGGGA*AATAAA*GTCAGAGGAATG

Supplementary 1



number	primer sequence
1	5'-GGAGGTGTATGTGAATGAGG-3'
2	5'-GGGGAAGCTTCACCCCGC TCCTCCCGGCTC-3'
3	5'-GGGGCTCGAGCCTGCTCCGCGCCTCCGTGT-3'
4	5'-CCTTAAAGAACTATAAGTCCCA-3'
5	5'-GAAGGAGAGACGCAGGAA-3'
6	5'-ACCTGCGGGCGGGGCCGGA-3'
7	5'-ACCTGCGGGCGGGGCCGGA-3'
8	5'-GAGGGAGGCGGGGTCAGAGG-3'
9	5'-AGAGGAATGAAGCGCGGAG-3'



	primer sequence
A	5'-AGCCCACCTGCAAATAAGGCCGGAGGGAGA-3'
A'	5'-TCTCCCTCCGGCCTTATTTGCAGGTGGGCT-3'
B	5'-GCAGACTCCTAAATAAGGTCCGGGTCACCA-3'
B'	5'-TGGTGACCCGGACCTTATTTA GGAGTCTGC-3'
C	5'-CGGCGCGAGGGAAATAAAGTCAGAGGAATG-3'
C'	5'-TTCATTCCTCTGACTTTATTTCCCTCGCGC-3'

Supplementary 2