ARTICLE
Journal of Cellular Biochemistry 110:1165–1174 (2010)

Journal of Cellular Biochemistry

Ganglioside GD1a Suppression of NOS2 Expression Via ERK1 Pathway in Mouse Osteosarcoma FBJ Cells

Ting Cao,[1] Tianyi Zhang,[1] Li Wang,[1] Lan Zhang,[1] Tomoko Adachi,[2] Toshinori Sato,[3] Sadako Yamagata,[1] and Tatsuya Yamagata[1*]

[1]*Laboratory of Tumor Biology and Glycobiology, Department of Life Sciences, Shenyang Pharmaceutical University, Shenyang 110016, People's Republic of China*

[2]*Bioscience and Biotechnology Center, Nagoya University, Nagoya 464-8601, Japan*

[3]*Department of Biosciences and Informatics, Keio University, Hiyoshi, Yokohama 223-8522, Japan*

ABSTRACT

Inducible nitric oxide synthase (NOS2) is over-expressed in a number of tumors and implicated in tumor growth and metastasis. Murine FBJ osteosarcoma-derived FBJ-S1 cells are poorly metastatic and express the ganglioside GD1a, whereas highly metastatic FBJ-LL cells only slightly express this ganglioside. The present study demonstrates that NOS2 is more highly expressed in FBJ-LL cells compared to FBJ-S1 cells. By manipulating GM2/GD2 synthase expression or adding exogenous GD1a, GD1a inversely regulated NOS2 at the transcriptional level. GT1b suppressed NOS2 to the same extent as GD1a. Silencing NOS2 inhibited proliferation, migration, and anchorage-independent growth of FBJ-LL cells, suggesting that the metastatic properties of FBJ-LL cells are associated with NOS2. MEK1/2 inhibitor (U0126) increased NOS2 expression, whereas GD1a treatment decreased it. Co-treating the cells with GD1a and U0126 blocked the inhibition of NOS2 expression, suggesting that the GD1a signal is mediated by ERK1/2. NOS2 expression increased when ERK1, but not ERK2, was silenced, and GD1a did not suppress NOS2 expression in cells treated with another MEK1/2 inhibitor PD98059, suggesting that ERK1 phosphorylation is indispensable for the GD1a signal suppressing NOS2. J. Cell. Biochem. 110: 1165–1174, 2010.

KEY WORDS: NOS2; FBJ OSTEOSARCOMA CELLS; GD1a; GT1b; ERK1/2

Inducible nitric oxide synthase (NOS2) has been identified in cytokine-activated mouse macrophages [Xie et al., 1992]. NOS2 is induced in response to a variety of pro-inflammatory cytokines in macrophages during host defense mechanisms, and it has been observed in many other cell types, such as endothelial and epithelial cells and a wide variety of tumor cells [Andrew and Mayer, 1999; Crowell et al., 2003]. Malignant neoplasms of the central nervous system express high levels of NOS2, and the increased expression correlates with vascularization and advanced tumor stages [Cobbs et al., 1995; Gallo et al., 1998]. Other studies have reported a correlation between strong NOS2 expression and a high incidence of metastasis in tumors [Thomsen et al., 1995; Lagares-Garcia et al., 2001; Song et al., 2002].

In our previous studies, poorly metastatic murine FBJ-S1 cells derived from FBJ virus induced osteosarcoma were found to express disialylated ganglioside GD1a, whereas highly metastatic FBJ-LL cells only slightly express this ganglioside [Hyuga et al., 1997]. The transfection of FBJ-LL cells with GM2/GD2 synthase (B4galnt1) cDNA results in increased GD1a expression [Hyuga et al., 1999]. Transfectants expressing a similar amount of GD1a as FBJ-S1 cells behave like FBJ-S1 cells and do not metastasize, whereas mock-transfectants were found to metastasize to the lung, liver, and adrenal gland, indicating that GD1a suppresses the metastatic ability of the tumor cells. GD1a has also been found to up-regulate caveolin-1 (Cav1) and stromal interaction molecule 1 (Stim1) [Wang et al., 2006] and to suppress the interaction between FBJ cells and vitronectin [Hyuga et al., 1999] and the expression of MMP-9 [Hu et al., 2007] and TNF-alpha [Wang et al., 2008]. Thus, GD1a plays a role in suppressing the metastatic ability of tumor cells through the regulation of these molecules.

A recent study showed that streptozotocin-induced diabetes causes an initial increase in the levels of NOS and

Additional Supporting Information may be found in the online version of this article.

Grant sponsor: Mizutani Foundation.

*Correspondence to: Prof. Tatsuya Yamagata, Laboratory of Tumor Biology and Glycobiology, Shenyang Pharmaceutical University, 103 WenHua Road, P.O. Box 29, Shenyang 110016, People's Republic of China.
E-mail: tcyamagata@cool.odn.ne.jp

Received 11 December 2009; Accepted 27 March 2010 • DOI 10.1002/jcb.22631 • © 2010 Wiley-Liss, Inc.
Published online 29 June 2010 in Wiley InterScience (www.interscience.wiley.com).

NADPH-diaphorase in the rat adrenal gland that is prevented by ganglioside treatment [Afework et al., 1996]. Also, nitric oxide (NO) plays an important role in the vasodilation induced by GM1 [Furian et al., 2008]. Although only a few studies have focused on the regulation of NOS2 by gangliosides, the relationship between gangliosides and other molecules related to vascularization, such as the VEGF family [Lang et al., 2001; Mukherjee et al., 2008], has been well elucidated. NOS2 is not only a key molecule involved in vascularization during pathological processes [Keynes and Garthwaite, 2004], but also a potent functional factor necessary for VEGF-induced vascularization [Ziche et al., 1997; Thil et al., 2005; Kim et al., 2008]. Therefore, we hypothesized that the ganglioside GD1a may regulate the expression of NOS2, leading to the suppression of cell invasion.

In this study we show that NOS2 expression is higher in highly metastatic FBJ-LL cells compared to the expression in poorly metastatic FBJ-S1 cells. We also provide evidence that NOS2 silencing inhibits the proliferation, migration, and anchorage-independent growth of FBJ-LL cells. The present study further shows that NOS2 expression is inversely regulated by GD1a at the transcriptional level in FBJ cells, and extracellular signal-regulated kinase 1/2 (MEK1/2) inhibitor U0126 blocks GD1a suppression of NOS2. We propose that the GD1a signal leading to NOS2 suppression is mediated by the MEK/ERK pathway. To the best of our knowledge, this paper is the first evidence that the GD1a signal is mediated by ERK1 phosphorylation.

MATERIALS AND METHODS

CELL LINES AND CULTURE

The highly metastatic murine osteosarcoma cell line FBJ-LL and the poorly metastatic cell line FBJ-S1 were produced from an FBJ virus-induced osteosarcoma in BALB/c mice [Yamagata et al., 1988]. FBJ-S1 cells express GM3 and GD1a, whereas FBJ-LL cells express GM3 but only a scant amount of GD1a [Hyuga et al., 1997]. LA5-30 cells were obtained by transfecting FBJ-LL cells with GM2/GD2 synthase cDNA, and mock-transfected M5 cells served as a control [Hyuga et al., 1999]. The cells were maintained in RPMI-1640 media as described previously [Wang et al., 2006]. To examine the effects of ganglioside on NOS2 expression, the cells were incubated with 50 μM GD1a in the absence of serum for 4 h and cultured with medium containing 5% FBS for an additional 20 h. When FBJ-LL cells or ERK-silenced transfectants were stimulated with GD1a, the cells were cultured in the serum-free medium for 6 h, and 50 μM GD1a was added to the medium in the presence or absence of ERK inhibitors.

CHEMICALS AND ANTIBODIES

Ganglioside GD1a from bovine brain was obtained from Wako (Osaka, Japan). MEK inhibitors U0126 and PD98059, and p38 inhibitor SB203580, were obtained from Sigma (USA). Jun-NH2-terminal kinase (JNK) inhibitor SP600125 was obtained from Biosource (USA). Rabbit anti-NOS2 antibody was purchased from Santa Cruz (USA). Mouse anti-β-actin antibody was purchased from Sigma (USA). Rabbit anti-ERK1/2 antibody was purchased from StressGen Biotechnologies Corp (Canada). Anti-phospho44/42 MAPK (Thr202/Tyr204) antibody, horseradish peroxidase (HRP)-linked anti-mouse IgG secondary antibody, and anti-rabbit IgG secondary antibody were purchased from Cell Signaling (USA).

RNA EXTRACTION AND RT-PCR

RT-PCR was used as previously described [Wang et al., 2006] to semi-quantitatively determine the levels of mRNA for the genes of interest. The RNeasy Mini kit was used to extract total RNA (Qiagen, Hilden, Germany), and the RT-PCR kit was from TAKARA Biotechnology Corporation (Dalian, China). Eukaryotic translation elongation factor 1 alpha 1 (Eef) mRNA was used as a control. The primers used for RT-PCR were designed by Primer 3 (v. 0.4.0) software and synthesized by Invitrogen (Shanghai, China). Primer sequences were as follows: Elongation factor 1 alpha 1 (Eef) sense 5′-CGCTGCTGGAAGCTTTGGAT-3′, antisense 5′-GGGGCCATCTTCCA-GCTTCT-3′; NOS2 sense 5′-GGCCAGGCTGGAAGCTGTAA-3′, anti-sense 5′-GCAAGACCAGAGGCAGCACA-3′; NOS1 sense 5′-GGGAC-CAGCCAAAGCAGAGA-3′, antisense 5′-GGCCTTGGAGCCAAACC-TCT-3′; NOS3 sense 5′-CCCAGCTGTGTCCAACATGC -3′, antisense 5′-TTCCAGGGGTCTGGCTGGTA-3′; ERK1 (Mapk3) sense 5′-ACGT-GCGCAAGACCAGAGTG-3′, antisense 5′-GTCGTGCTCAGGGTCAG-CAA-3′; ERK2 (Mapk1) sense 5′-GCCTTCCAACCTCCTGCTGA-3′, antisense 5′-CAGGTATGGGTGGGCCAGAG-3′; GM2/GD2 synthase (B4galnt1) sense 5′-ACCTGGCGGTGTCCCAAGTA-3′, antisense 5′-TCAGCTCCTGGGTCCTTTGC-3′.

siRNA TRANSFECTION

Transfections of siRNA vectors targeting NOS2 and ERK1/2 into FBJ-LL cells were performed as described previously [Wang et al., 2006]. The sequences were made to constitute a retroviral vector with neomycin resistance at TAKARA Biotechnology Corporation (Dalian, China). Plasmids were transfected into FBJ-S1 cells in the presence of Fugene (Roche, USA) as specified by the manufacturer. Target sequences for NOS2, ERK1 (Mapk3), and ERK2 (Mapk1) were 5′-CTGTAGCACAGCACAGGAAAT, 5′-TGACCACATCTGCTACTTC-CT, and 5′-AGCATTACCTTGACCAGCTGA, respectively.

WESTERN BLOTTING

Cells (1×10^6) were lysed in 100 μl lysis buffer at 4°C for 30 min and boiled at 100°C for 5 min. An aliquot of the lysate was loaded on a SDS–polyacrylamide gel, with the gel concentration depending on the molecule weight of the target protein. Following electrophoresis, the gel was blotted on a PVDF membrane (Amersham Hybond-P membrane, Amersham, USA). The membrane was incubated with the first antibody diluted 1:2,000, followed by incubation with the HRP-conjugated secondary antibody (1:5,000 dilution). Western blots were visualized by ECL Western blotting detection reagents (Amersham Biosciences). Lanes were scanned and the optical density determined by the Bio-profile Bio 1D image analyzer (Vilber Lourmat, Marne-la-Vallee, France).

dsRNA TRANSFECTION

Cells (1×10^6) were seeded in 6 cm dishes. The following day, 80 μl Opti-MEM (GIBCO, Invitrogen Corporation, NY) was mixed with 3 μl X-tremeGENE (Roche) and 1.2 μl dsRNA for 5 min in a 96-well plate. The mixture was kept still at room temperature for 30 min and

transferred to the cells containing 1.2 ml serum-free medium. After 4 h, the medium was changed to normal medium, and 20 h later the cells were extracted for mRNA, lipid, or protein analysis. The two sequences of one strand of double-stranded siRNA (GenePharma Corporation, Shanghai, China) directed to GM2/GD2 synthase were 5′-GAGAUCUCGGGCUACGCUATT and 5′-CUGAUGUUGUUGUG-GAUCATT.

GANGLIOSIDE EXTRACTION AND HPTLC

Glycolipids were extracted from 2×10^6 cells using 1 ml chloroform/methanol 1:1 (v/v), followed by a second extraction in 1 ml chloroform/methanol 2:1 (v/v), and processed as described previously [Wang et al., 2006].

NO PRODUCTION ANALYSIS

NO production was estimated by measuring nitrate/nitrite in the cell culture medium. Culture medium (100 μl) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid) at room temperature for 10 min. The absorbance at 540 nm was monitored by an MTP-120 microplate reader (Corona Electric, Japan). The total nitrate/nitrite concentration was determined by a standard curve prepared with sodium nitrite.

WST ASSAY

Cell proliferation was measured by WST assay according to the manufacturer's protocol. Briefly, cells were cultured for the time indicated, and 1:10 volume of the Cell Counting Kit (WST, Dojindo, Japan) solution was added and the cells incubated for another 3 h. The number of living cells was measured in a 96-well plate using the microplate reader at 450 nm with a reference filter of 660 nm.

STATISTICAL ANALYSIS

All values are given as the mean ± SD. Statistical analyses were performed using the Student's *t*-test available in Microsoft Excel, and the level of significance is indicated in figures when more than three independent experiments were performed.

RESULTS

NOS2 EXPRESSION IS POSSIBLY IN INVERSE RELATION TO GD1a IN FBJ CELLS

NOS2 mRNA and protein in FBJ cells were assessed by RT-PCR and Western blot, respectively. NOS2 expression was much higher in highly metastatic FBJ-LL cells than poorly metastatic FBJ-S1 cells (Fig. 1A,B). NOS2 expression was reflected in NO production, as assessed by Griess reagent (Fig. 1C). NO production in FBJ-LL cells was several times higher than in FBJ-S1 cells, implying that NOS2 is related to the metastatic ability of FBJ cells by the production of NO gas. There are two other NO synthases responsible for NO production, but NOS1 and NOS3 mRNA expression was low (both were detectable at 34 cycles) compared to NOS2 (21 cycles) (see Fig. S1 in Supplementary Material); thus, we focused on NOS2 in the present study. In order to further evaluate the role of NOS2 in FBJ cells, FBJ-LL transfectants were obtained in which NOS2 expression was suppressed by vector-based siRNA directed to NOS2 (Fig. S2A).

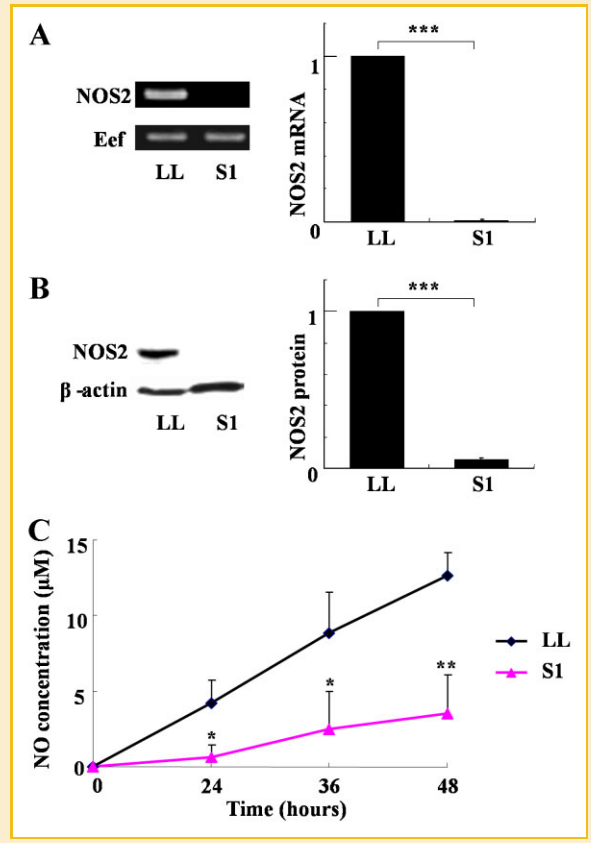

Fig. 1. NOS2 was highly expressed in highly metastatic FBJ-LL cells compared to poorly metastatic FBJ-S1 cells. FBJ-LL and FBJ-S1 cells were cultured under normal conditions, and total RNA or protein was extracted. A: NOS2 mRNA expression was detected by RT-PCR using eukaryotic translation elongation factor 1 alpha 1 (Eef) as a standard. Left, representative results of PCR; right, densitometric analysis with the ratio of NOS2/Eef in FBJ-LL cells considered as 1. B: NOS2 protein expression was determined by Western blot using β-actin as a standard. The densitometric analysis is shown on the right, with the ratio of NOS2/β-actin in FBJ-LL cells considered as 1. C: FBJ-LL and FBJ-S1 cells were cultured for 24, 36, or 48 h, and aliquots of the conditioned medium were assayed for NO by Griess reagent. In this figure, data are the mean of three independent experiments, and the error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ where P values are calculated against the control.

Among the transfectants, cells with significantly suppressed NOS2 mRNA (Fig. S2A) were assayed for cell motility using cell growth (Fig. S2B), Transwell (Fig. 2C), wound healing (Fig. S2D), and anchorage-independent growth by cultivating the cells in soft agar (Fig. S2E). All of these features of FBJ-LL cells were significantly inhibited following NOS2 silencing, indicating that NO production by NOS2 is related to the metastatic ability of FBJ-LL cells. Taken together, these results suggest that NOS2 expression that may be inversed to GD1a contents plays an important role in the activity of FBJ cells.

NOS2 EXPRESSION IS INVERSELY REGULATED BY GD1a

The FBJ-LA5-30 cell line was obtained by transfecting FBJ-LL cells with GM2/GD2 synthase cDNA, and FBJ-M5 was a mock-transfectant [Hyuga et al., 1999]. FBJ-LA5-30 cells expressed GD1a

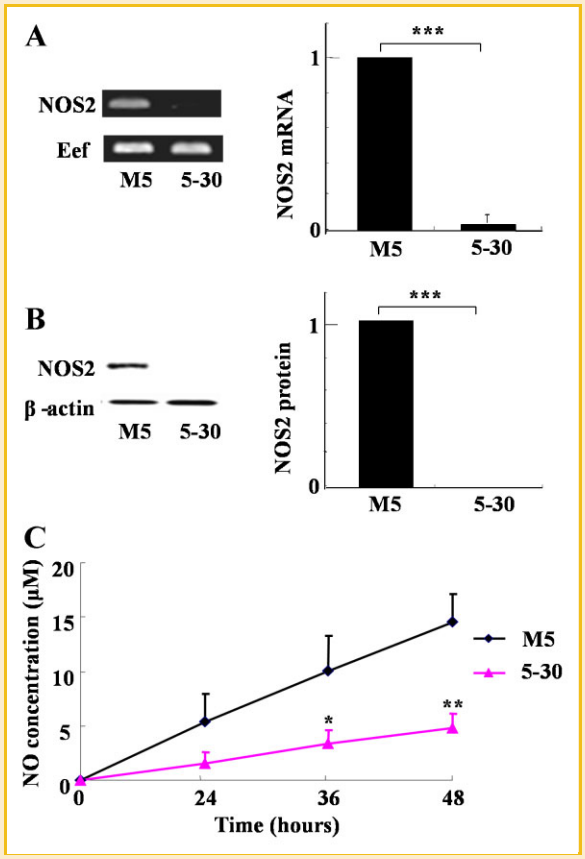

Fig. 2. NOS2 was suppressed by over-expression of GD1a in FBJ-LL cells. Previously obtained LA5-30 cells (GM2/GD2 synthase cDNA-transfected FBJ-LL cells) and FBJ-M5 cells (mock-transfected) were cultured under normal conditions and total RNA or protein extracted. A: NOS2 mRNA expression was detected by RT-PCR using Eef as a standard. Right, densitometric analysis with the NOS2/Eef ratio in M5 cells considered as 1; left, representative PCR result. B: The NOS2 protein level was determined by Western blot using β-actin as a standard. The densitometric analysis is shown at the right. C: FBJ-M5 and LA5-30 cells were cultured under normal conditions for 24, 36, or 48 h, and the conditioned medium was analyzed for NO by Griess reagent. Data in this figure are the mean of three independent experiments, the error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

similarly to FBJ-S1 cells. NOS2 mRNA and protein were decreased in LA5-30 cells compared to M5 cells; NOS2 expression in LA5-30 cells was as low as the expression measured in FBJ-S1 cells (Fig. 2A,B). Also, NO production in LA5-30 cells was less than in M5 cells (Fig. 2C). To confirm if GD1a down-regulates NOS2 in FBJ cells, the double stranded siRNA directed to GM2/GD2 synthase (B4galnt1) was administered to GD1a-rich FBJ-S1 cells to decrease GD1a expression. Ganglioside pattern analysis revealed that transfectants contained less GD1a compared to the control cells; however, the levels of other gangliosides, such as GM3, did not change (Fig. 3A). The expression of GM2/GD2 synthase mRNA decreased in double-stranded siRNA-transfected S1 cells, whereas the expression of NOS2 inversely increased (Fig. 3B), suggesting that NOS2 expression is regulated by endogenous GD1a at the transcriptional level. In order to determine whether exogenous GD1a affects NOS2 expression, FBJ-LL cells were cultivated in serum-free medium in the presence of GD1a. Treating the FBJ-LL cells with 50 μM GD1a for 24 or 48 h decreased NOS2 mRNA and protein (Fig. 4A,B). Exogenous GD1a down-regulating NOS2 expression is consistent with the finding that endogenous GD1a suppresses NOS2 expression. The suppression of NOS2 expression in GD1a-treated FBJ-LL cells was reflected in decreased NO production (Fig. 4C). The presence of GD1a was confirmed to have no effect on NO determination (data not shown). NO production was lower in the GD1a-treated cells compared to control cells (Fig. 4C). The decrease in NO production may have been due to decreased cell number. Cell number was determined by WST assay after treating FBJ-LL cells with GD1a for 48 h. The NO concentration was divided by the cell number obtained after 48 h incubation; GD1a treatment decreased NO production per cell (Fig. 4D). These results demonstrate that the exogenous treatment of FBJ cells with GD1a suppresses NOS2 expression, resulting in reduced NO production. Taken together, the data indicate that GD1a regulates NOS2 expression in FBJ cells. Because NOS2 is suppressed at both the mRNA and protein levels, GD1a targets NOS2 at the transcriptional level in FBJ cells.

NeuAc-Gal-GalNAc SEEMS TO BE ESSENTIAL FOR GANGLIOSIDE SUPPRESSION OF NOS2 EXPRESSION

GD1a markedly inhibited NOS2 expression in FBJ cells. To investigate whether other gangliosides exert a similar effect, FBJ-LL cells were treated with gangliosides GM3, GM1a, GD1b, or GT1b under the same conditions as GD1a. Monosialylated gangliosides GM3 and GM1a had no effect. GD1b had no effect on NOS2, whereas GT1b decreased NOS2 expression to the same extent as GD1a (Fig. 5). Effective GD1a has one sialyl residue and ineffective GD1b has two sialyl residues at the internal Gal, as does GT1b. Thus, sialylation at the internal Gal is not essential. GD1a and GT1b share the structure NeuAcα2,3-Galβ1,3-GalNAcβ, whereas ineffective GM3 possesses NeuAcα 2,3-Galβ1,4-Glcβ, implying that NeuAcα2,3-Galβ1,3-GalNAcβ may be the functional key for ganglioside regulation of NOS2 expression.

ERK1/2 PATHWAYS ARE INVOLVED IN NOS2 REGULATION

We have shown that the addition of exogenous GD1a to FBJ-LL cells suppresses NOS2 (Fig. 4). The regulation of NOS2 has been reported to be mainly mediated by the mitogen-activated protein kinase (MAPK) pathway [Bhat et al., 1998; Wright et al., 2006]. To determine which MAPK pathway mediates the GD1a signal to suppress NOS2, FBJ-LL cells were treated with 50 μM GD1a, 10 μM U0126 (MEK1/2 inhibitor), 20 μM SB203580 (p38 inhibitor), 20 μM SP600125 (JNK inhibitor), or GD1a in the presence of any of the aforementioned inhibitors for 24 h. GD1a suppressed NOS2 expression to 64%, U0126 increased its expression to 149%, and GD1a plus U0126 increased expression to 126% (Fig. 6). The elevation of NOS2 expression in the presence of U0126 suggests that ERK1/2 inhibits NOS2 expression in FBJ-LL cells. MEK inhibitor cancelled the effect of GD1a (Fig. 6), suggesting that the suppressive effect of GD1a on NOS2 expression is mediated by the ERK1/2 pathway. The p38 inhibitor up-regulated NOS2, whereas JNK inhibitor down-regulated NOS2 expression. However, because these

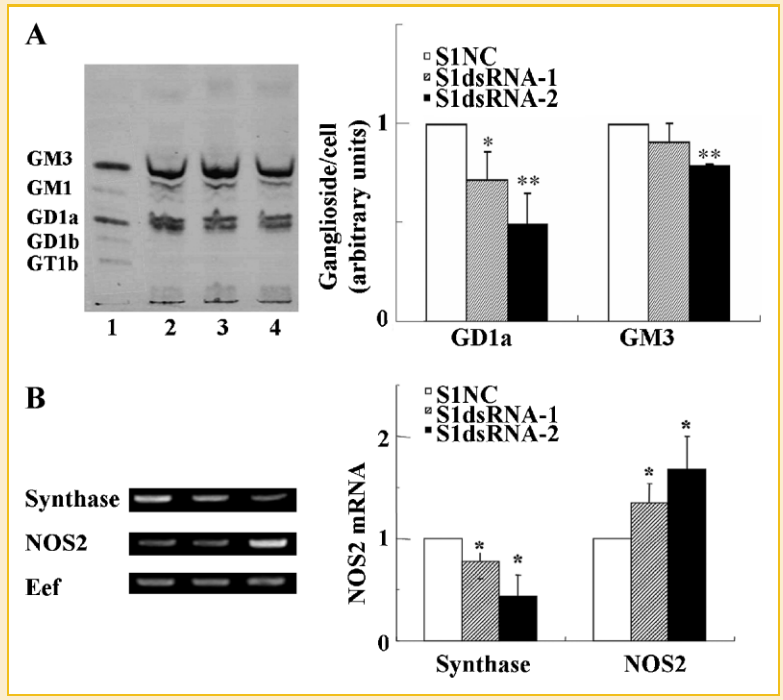

Fig. 3. GM2/GD2 synthase dsRNA transfection in FBJ-S1 cells increased NOS2. FBJ-S1 cells were transfected with GM2/GD2 dsRNA and RNA or lipid extracted. A: The ganglioside pattern was analyzed by HPTLC after transfection for 48 h. Lane 1, standard ganglioside mixture obtained from bovine brain in addition to GM3: lane 2, S1 cells transfected with dsRNA nonspecific control; and lanes 3 and 4, S1 cells transfected with GM2/GD2 synthase dsRNA sequences 1 and 2, respectively. HPTLC analysis was performed once. A three times and an average of the densitometric analysis is shown at the right, where S1NC stands for FBJ-S1 cells transfected with scrambled sequence. The error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$. B: GM2/GD2 synthase and NOS2 mRNA expression was investigated by RT-PCR after transfection for 24 h using Eef as a standard. Left, representative PCR result; right, densitometric analysis of mRNA expression. The data is the mean of three independent experiments. The error bars represent SD; $^{*}P < 0.05$.

two inhibitors did not affect GD1a suppression of NOS2, the p38 and JNK pathways are not shown to be involved in the regulation of NOS2 by GD1a. We further investigated which ERK1/2 molecule is involved in NOS2 regulation by using ERK1 or ERK2 siRNA vector-transfected FBJ-LL monoclonal cells. NOS2 expression was increased in ERK1-silenced FBJ-LL monoclonal M3-14, M3-17, and M3-18 cells at both the mRNA (Fig. S3A) and protein (Fig. S3B) levels compared to scrambled sequence transfected cells, whereas NOS2 expression did not change in the ERK2-silenced monoclonal cells M1-3, M1-4, and M1-7, suggesting that ERK1, but not ERK2, inhibits NOS2 expression in FBJ cells.

GD1a SUPPRESSION OF NOS2 IS MEDIATED BY ERK1 PHOSPHORYLATION

To further investigate the role of ERK1/2 in the regulation of NOS2 by GD1a, ERK1 or ERK2 silenced FBJ-LL cells were treated with GD1a. GD1a suppressed NOS2 expression to 57–60% in scrambled sequence transfected LL cells (Fig. 7A,B). GD1a suppressed NOS2 mRNA in ERK2-silenced FBJ-LL monoclonal cells M1-3, M1-4, and M1-7 to 63%, 49%, and 60%, respectively (average 57%; Fig. 7A), suggesting that ERK2 is not involved in the GD1a signal. However, in the ERK1-silenced FBJ-LL monoclonal cells, NOS2 expression following GD1a treatment was 79%, 76%, and 110% (average 88%) in M3-14, M3-17, and M3-18 cells, respectively, compared to the control (Fig. 7B). GD1a failed to suppress NOS2 expression in ERK1-silenced FBJ-LL cells, suggesting that GD1a down-regulates NOS2 via the ERK1, but not ERK2, pathway in FBJ cells. The phosphorylation level of ERK1/2 was assayed in ERK1-silenced FBJ-LL cells incubated with GD1a for 5 min. In these transfectants, the extent of ERK1 phosphorylation was proportional to the remaining ERK1 protein. In M3-18 cells in which the GD1a signal failed to suppress NOS2 expression (Fig. 7B), the amount of ERK1 protein was suppressed and ERK1 phosphorylation was nearly undetectable, indicating that ERK1 phosphorylation is essential for GD1a suppression of NOS2 expression (Fig. 7C).

PD98059, another inhibitor of ERK phosphorylation, was used to further confirm this notion by treating FBJ-LL cells with GD1a and varying concentrations of PD98059. As the concentration of PD98059 increased, NOS2 expression proportionally increased and more evidently cancelled the GD1a suppression of NOS2 (Fig. 8A). Under the conditions adopted, we confirmed that ERK1 phosphorylation is suppressed by PD98059 (Fig. 8B). The phosphorylation of ERK1 by GD1a was proportionally suppressed by increasing concentrations of PD98059, further supporting the notion that ERK1 phosphorylation is indispensable for the suppression of NOS2 expression by GD1a in FBJ-LL cells (Fig. 8A,B). Taken together, the results indicate that GD1a inhibits NOS2 expression by activating ERK1 phosphorylation. Thus, the ERK1 pathway plays an important role in NOS2 suppression as a mediator of the GD1a signal in FBJ-LL cells.

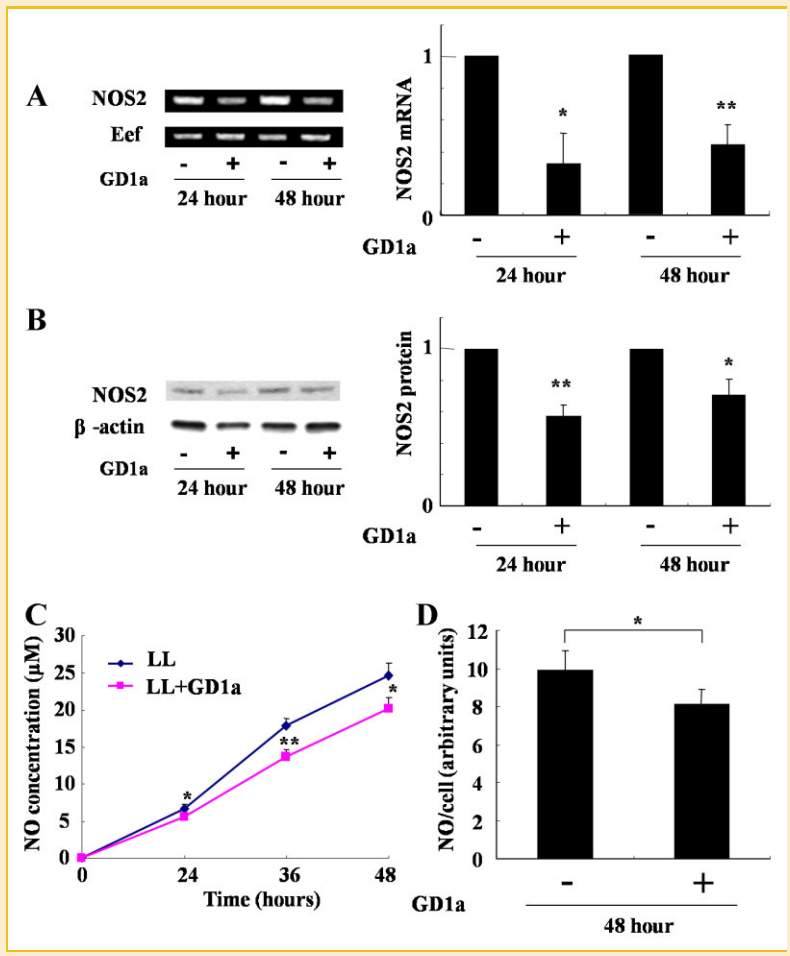

Fig. 4. Exogenous GD1a suppressed NOS2 expression and NO production in FBJ-LL cells. FBJ-LL cells were incubated with 50 μM GD1a for 24 or 48 h. Total RNA or protein was extracted and NOS2 expression determined by (A) RT-PCR using Eef as a standard and (B) Western blot using β-actin as a standard. C: FBJ-LL cells were treated with 50 μM GD1a for 24, 36, or 48 h and the conditioned medium analyzed by Griess reagent. D: NO production was normalized by the cell number at 48 h. Following the experiment shown in (C), NO concentrations at 48 h were divided by the cell number (measured by WST assay at O.D. 450 nm). Data in this figure are the mean of three independent experiments. The error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$.

DISCUSSION

NO is produced by three species of NOS, but FBJ cells barely expressed NOS1 and NOS3. Thus, NOS2 expression was focused on in two FBJ cell lines with different metastatic capacities. FBJ-S1 cells are not metastatic and rich in GD1a, whereas FBJ-LL cells are highly metastatic and mostly deficient in GD1a. FBJ-LL was found to be rich in NOS2, whereas FBJ-S1 expressed a very small amount of NOS2. The expression of NOS2 mRNA was reflected in the amount of protein produced and amount of NO detected by Griess reagent. The

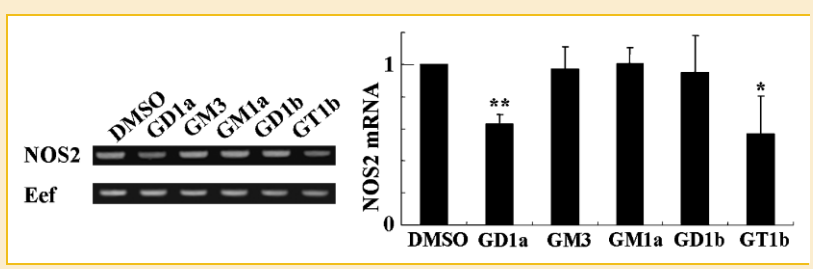

Fig. 5. Gangliosides GT1b and GD1a suppressed NOS2 to the same extent. FBJ-LL cells were incubated with 50 μM GD1a, GM3, GM1a, GD1b, or GT1b in serum-free medium for 24 h. Total RNA was extracted and NOS2 mRNA expression investigated by RT-PCR using Eef as a standard. Left, representative PCR; right, densitometric analysis with the ratio of NOS2/Eef in DMSO control cells considered as 1. Data are the mean of three independent experiments. Error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$.

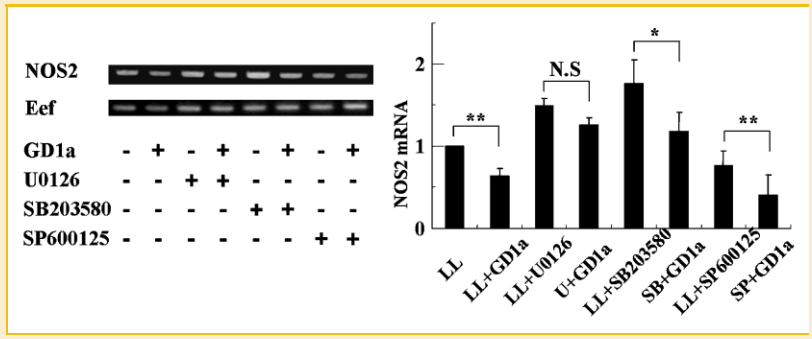

Fig. 6. ERK1/2 pathway seems to be involved in NOS2 regulation. FBJ-LL cells were pre-treated with 10 μM U0126, 20 μM SB203580, or 20 μM SP600125 for 1 h, and then fed 50 μM GD1a and incubated for 24 h. Total RNA was extracted and NOS2 expression investigated by RT-PCR using Eef as a standard. Left, representative PCR result; right, densitometric analysis with the ratio of NOS2/Eef in control FBJ-LL cells considered as 1. Data are the mean of three independent experiments. Error bars represent SD; $^{*}P < 0.05$, $^{**}P < 0.01$, NS, non-significant.

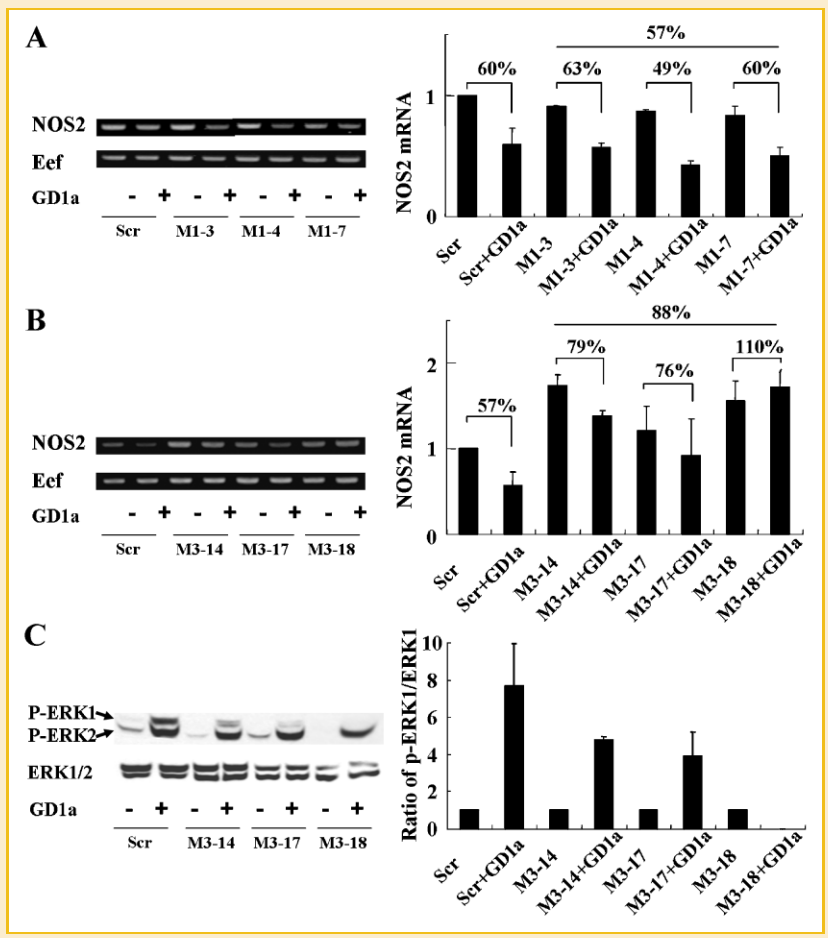

Fig. 7. ERK1 phosphorylation was indispensable to the suppression of NOS2 by GD1a in ERK-silenced transfectants. A,B: The effects of ERK1/2 silencing on the GD1a signal. Scrambled sequence-transfected, ERK1-silenced, or ERK2-silenced FBJ-LL cells were treated with 50 μM GD1a for 24 h and RNA extracted. NOS2 mRNA expression was detected by RT-PCR using Eef as a standard. The ratio of NOS2/Eef in the scrambled control was expressed as 1. A representative PCR result is given at the left. C: Phosphorylation of ERK1/2 by GD1a in the ERK1-silenced cells. ERK1-silenced cells were treated with 50 μM GD1a in serum-free medium for 5 min. Protein was extracted and analyzed by Western blot using total ERK as a standard. The ratio of ERK1 phosphorylation/ERK1 in the scrambled control was expressed as 1. A representative Western blot is given at the left. Data presented in C are the mean of three independent experiments. The error bars represent SD.

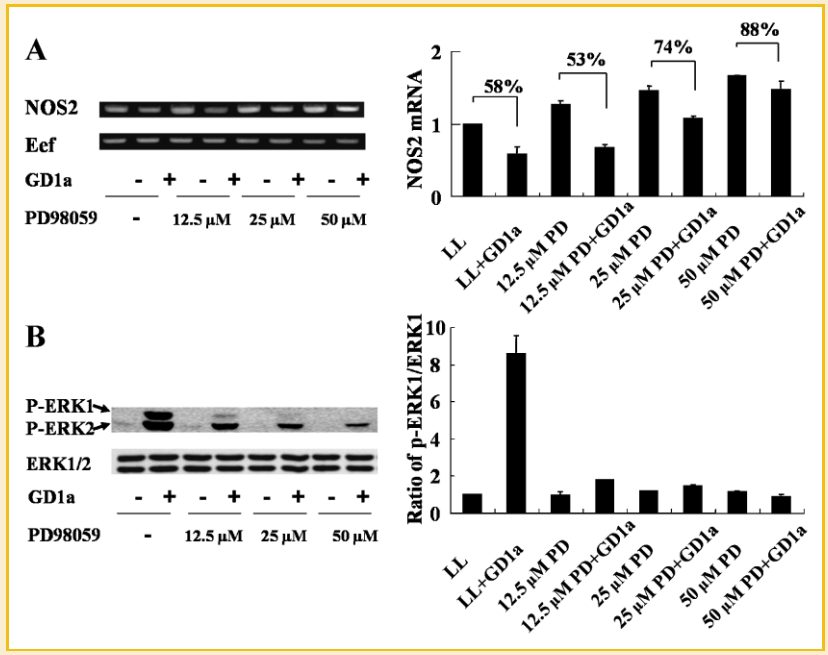

Fig. 8. MEK inhibitor showed that ERK1 phosphorylation is indispensable for the suppression of NOS2 by GD1a. A: FBJ-LL cells were pre-treated with 12.5, 25, or 50 μM PD98059 for 1 h, and then fed 50 μM GD1a and incubated for 24 h. Total RNA was extracted and NOS2 expression investigated by RT-PCR using Eef as a standard. Left, the result of PCR; right, densitometric analysis with the ratio of NOS2/Eef in FBJ-LL cells considered as 1. B: FBJ-LL cells cultured in serum-free medium for 6 h were treated with 12.5, 25, or 50 μM PD98059 for 1 h and treated with 50 μM GD1a for 5 min. Protein was extracted and analyzed by Western blot using total ERK as a standard. Left, representative results of Western blot; right, densitometric analysis. Data presented are the mean of three independent experiments; the error bars represent SD.

expression of NOS2 was shown to be related to the activity of FBJ cells (Fig. S2). NOS2 was shown to be under the inverse control of GD1a; increased GD1a (by introducing GM2/GD2 synthase or by exogenous addition of GD1a) suppressed NOS2 expression, whereas decreased GD1a (by silencing GM2/GD2 synthase) resulted in increased NOS2. The change in NOS2 expression was reflected in the protein levels and NO production. Thus, we unequivocally showed that GD1a regulates NOS2 at the transcriptional level in FBJ cells.

MAPKs are important regulators of a broad range of genes involved in cellular responses to inflammatory and stress signals [Cobb and Goldsmith, 1995]. Three major mammalian MAPK pathways have been identified: ERK1/2, JNK, and p38. The ERK1/2 pathway is regulated primarily by growth factors and tumor promoters, and the JNK and p38 pathways are activated by stress and inflammatory agents, including IL-1. The role of MAPK pathways in the cytokine (LPS, IL-1, and TNFα) induced expression of NOS2 has been well studied, and the functions of ERK1/2, p38 MAPK, and JNK in the regulation of NOS2 are diverse, depending on different cytokine inducers and cell lines. In many cases, the pathways have been considered to promote NOS2 expression [Da Silva et al., 1997; Bhat et al., 1998; Hua et al., 2002; Wright et al., 2006; Korhonen et al., 2007] under cytokine stimulation. However, the ERK1/2 and p38 MAPK pathways have also displayed a suppression of NOS2 in some cases [Chan and Riches, 2001]. Thus, the role of MAPK pathways in NOS2 regulation is complicated.

In the case of FBJ-LL cells in the present study, ERK1/2 and p38 were shown to be involved in the down-regulation of NOS2, whereas JNK was involved in its up-regulation. GD1a was shown to suppress NOS2 expression, and the effect of GD1a was blocked by treating cells with ERK1/2 inhibitor. The inhibitors of p38 and JNK did not impair the GD1a-mediated effects, indicating that ERK1/2 is the pathway via which the GD1a signal suppresses NOS2. Specifically, the phosphorylation of ERK1 was shown to be involved in the GD1a signal.

What causes the enhanced production of NOS in FBJ-LL cells is not clear, but autocrine TNFα is likely responsible for NOS2 over-expression because TNFα production in FBJ-LL cells is higher than in FBJ-S1 cells [Wang et al., 2008], and treating FBJ cells with TNFα increases NOS2 [L. Zhang, unpublished]. The present study demonstrated that the expression of GD1a in FBJ-S1 cells may explain the low level of NOS2 production in these cells, but the mechanism of how the GD1a signal negatively regulates NOS2 mRNA in FBJ-S1 cells is not currently clear. In the FBJ-LL cells, which lack GD1a, the GD1a signal was shown to be mediated through the ERK1 signaling pathway, but not ERK2. This will be confirmed in our following study on manipulation of MEK2 that is implicated in phosphorylation of ERK1.

Gangliosides are associated with cell growth, cell adhesion, cell differentiation, cell motility, tumorigenesis, and tumor metastasis, and changes in ganglioside expression in tumor cells are strongly related to the metastatic potential [Hakomori, 1996]. GM1a and GM3 have been shown to suppress EGF- and PDGF-derived mitogenesis [Bremer et al., 1984] by reducing the phosphorylation of the receptor upon ligand binding. GM3 inhibits the tyrosine kinase activity of EGFR through carbohydrate to carbohydrate interactions between the *N*-glycans with GlcNAc termini on EGFR and oligosaccharides

on GM3 [Kawashima et al., 2009], and GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis via the direct interaction of GM3 with VEGFR-2, blocking receptor activation [Chung et al., 2009]. GM3 and/or GM2 are shown to inhibit integrin-dependent tumor cell motility via the formation of a ganglioside/tetraspanin/integrin receptor complex responsible for the negative regulation of c-Src [Mitsuzuka et al., 2005] and c-Met [Todeschini et al., 2007; Todeschini et al., 2008]. Recently, it was found that up-regulation of GM3 synthase resulted in suppression of tumor cell motility, induction of caveolin-1 expression and enhanced expression of the inactive form of c-Src [Prinetti et al., 2010]. The effects of gangliosides on the growth factor are diverse; silencing of GM3 synthase suppressed lung metastasis of murine breast cancer cells [Gu et al., 2008]. In the case of human normal dermal fibroblasts, GD1a has been found to increase EGF-receptor dimerization and enhance receptor signaling in the presence or absence of the ligand [Liu et al., 2004]. GD1a has been shown to suppress FBJ cell metastasis [Hyuga et al., 1999] and bind c-Met to suppress signal transduction following HGF binding in FBJ-LL cells; in GD1a-rich cells, the phosphorylation of c-MET by HGF is suppressed compared to FBJ-LL cells [Hyuga et al., 2001]. However, whether the regulation of c-Met by GD1a is related to the metastatic capacity of the cells remains to be elucidated.

GT1b was found to be as effective as GD1a in the up-regulation of caveolin-1 in FBJ-LL cells [Wang et al., 2006], which was also the case with the suppression of NOS2 in FBJ-cells. GD1b failed to have any effect on either caveolin-1 or NOS2 expression. Gangliosides GD1a and GT1b share the common saccharide structure NeuAcα2-3-Galβ1-3-GalNAc; thus, this structure is indispensable for ganglioside interaction with yet unidentified molecules to exert ganglioside function. We have been, and are still currently, looking for the molecules that may bind GD1a to convey the GD1a signal to the cells to elucidate the mechanism by which the ganglioside GD1a regulates metastasis.

ACKNOWLEDGMENTS

This work was supported in part by funds from the Mizutani Foundation for Glycoscience (to T.Y.).

REFERENCES

Afework M, Lincoln J, Belai A, Burnstock G. 1996. Increase in nitric oxide synthase and NADPH-diaphorase in the adrenal gland of streptozotocin-diabetic Wistar rats and its prevention by ganglioside. Int J Dev Neurosci 14:111–123.

Andrew PJ, Mayer B. 1999. Enzymatic function of nitric oxide synthases. Cardiovasc Res 43:521–531.

Bhat NR, Zhang P, Lee JC, Hogan EL. 1998. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J Neurosci 18:1633–1641.

Bremer EG, Hakomori S, Bowen-Pope DF, Raines E, Ross R. 1984. Ganglioside-mediated modulation of cell growth, growth factor binding, and receptor phosphorylation. J Biol Chem 259:6818–6825.

Chan ED, Riches DW. 2001. IFN-gamma+LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. Am J Physiol Cell Physiol 280:C441–C450.

Chung TW, Kim SJ, Choi HJ, Kim KJ, Kim MJ, Kim SH, Lee HJ, Ko JH, Lee YC, Suzuki A, Kim CH. 2009. Ganglioside GM3 inhibits VEGF/VEGFR-2-mediated angiogenesis: Direct interaction of GM3 with VEGFR-2. Glycobiology 19:229–239.

Cobb MH, Goldsmith EJ. 1995. How MAP kinases are regulated. J Biol Chem 270:14843–14846.

Cobbs CS, Brenman JE, Aldape KD, Bredt DS, Israel MA. 1995. Expression of nitric oxide synthase in human central nervous system tumors. Cancer Res 55:727–730.

Crowell JA, Steele VE, Sigman CC, Fay JR. 2003. Is inducible nitric oxide synthase a target for chemoprevention? Mol Cancer Ther 2:815–823.

Da Silva J, Pierrat B, Mary JL, Lesslauer W. 1997. Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. J Biol Chem 272:28373–28380.

Furian AF, Oliveira MS, Magni DV, Souza MA, Bortoluzzi VT, Bueno LM, Royes LF, Mello CF. 2008. L-NAME prevents GM1 ganglioside-induced vasodilation in the rat brain. Neurochem Int 53:362–369.

Gallo O, Masini E, Morbidelli L, Franchi A, Fini-Storchi I, Vergari WA, Ziche M. 1998. Role of nitric oxide in angiogenesis and tumor progression in head and neck cancer. J Natl Cancer Inst 90:587–596.

Gu Y, Zhang J, Mi W, Yang J, Han F, Lu X, Yu W. 2008. Silencing of GM3 synthase suppresses lung metastasis of murine breast cancer cells. Breast Cancer Res 10:R1.

Hakomori S. 1996. Tumor malignancy defined by aberrant glycosylation and sphingo(glyco)lipid metabolism. Cancer Res 56:5309–5318.

Hu D, Man Z, Wang P, Tan X, Wang X, Takaku S, Hyuga S, Sato T, Yao X, Yamagata S, Yamagata T. 2007. Ganglioside GD1a negatively regulates matrix metalloproteinase-9 expression in mouse FBJ cell lines at the transcriptional level. Connect Tissue Res 48:198–205.

Hua LL, Zhao ML, Cosenza M, Kim MO, Huang H, Tanowitz HB, Brosnan CF, Lee SC. 2002. Role of mitogen-activated protein kinases in inducible nitric oxide synthase and TNFalpha expression in human fetal astrocytes. J Neuroimmunol 126:180–189.

Hyuga S, Yamagata S, Tai T, Yamagata T. 1997. Inhibition of highly metastatic FBJ-LL cell migration by ganglioside GD1a highly expressed in poorly metastatic FBJ-S1 cells. Biochem Biophys Res Commun 231:340–343.

Hyuga S, Yamagata S, Takatsu Y, Hyuga M, Nakanishi H, Furukawa K, Yamagata T. 1999. Suppression by ganglioside GD1A of migration capability, adhesion to vitronectin and metastatic potential of highly metastatic FBJ-LL cells. Int J Cancer 83:685–691.

Hyuga S, Kawasaki N, Hyuga M, Ohta M, Shibayama R, Kawanishi T, Yamagata S, Yamagata T, Hayakawa T. 2001. Ganglioside GD1a inhibits HGF-induced motility and scattering of cancer cells through suppression of tyrosine phosphorylation of c-Met. Int J Cancer 94:328–334.

Kawashima N, Yoon SJ, Itoh K, Nakayama K. 2009. Tyrosine kinase activity of epidermal growth factor receptor is regulated by GM3 binding through carbohydrate to carbohydrate interactions. J Biol Chem 284:6147–6155.

Keynes RG, Garthwaite J. 2004. Nitric oxide and its role in ischaemic brain injury. Curr Mol Med 4:179–191.

Kim CO, Huh AJ, Kim MS, Chin BS, Han SH, Choi SH, Jeong SJ, Choi HK, Choi JY, Song YG, Kim JM. 2008. LPS-induced vascular endothelial growth factor expression in rat lung pericytes. Shock 30:92–97.

Korhonen R, Linker K, Pautz A, Forstermann U, Moilanen E, Kleinert H. 2007. Post-transcriptional regulation of human inducible nitric-oxide synthase expression by the Jun N-terminal kinase. Mol Pharmacol 71:1427–1434.

Lagares-Garcia JA, Moore RA, Collier B, Heggere M, Diaz F, Qian F. 2001. Nitric oxide synthase as a marker in colorectal carcinoma. Am Surg 67:709–713.

Lang Z, Guerrera M, Li R, Ladisch S. 2001. Ganglioside GD1a enhances VEGF-induced endothelial cell proliferation and migration. Biochem Biophys Res Commun 282:1031–1037.

Liu Y, Li R, Ladisch S. 2004. Exogenous ganglioside GD1a enhances epidermal growth factor receptor binding and dimerization. J Biol Chem 279:36481–36489.

Mitsuzuka K, Handa K, Satoh M, Arai Y, Hakomori S. 2005. A specific microdomain (''glycosynapse 3'') controls phenotypic conversion and reversion of bladder cancer cells through GM3-mediated interaction of alpha3-beta1 integrin with CD9. J Biol Chem 280:35545–35553.

Mukherjee P, Faber AC, Shelton LM, Baek RC, Chiles TC, Seyfried TN. 2008. Thematic review series: Sphingolipids. Ganglioside GM3 suppresses the proangiogenic effects of vascular endothelial growth factor and ganglioside GD1a. J Lipid Res 49:929–938.

Prinetti A, Aureli M, Illuzzi G, Prioni S, Nocco V, Scandroglio F, Gagliano N, Tredici G, Rodriguez-Menendez V, Chigorno V, Sonnino S. 2010. GM3 synthase overexpression results in reduced cell motility and in caveolin-1 upregulation in human ovarian carcinoma cells. Glycobiology 20:62–77.

Song ZJ, Gong P, Wu YE. 2002. Relationship between the expression of iNOS, VEGF, tumor angiogenesis and gastric cancer. World J Gastroenterol 8:591–595.

Thil MA, Vince V, Veraart C, Delbeke J, Colin IM. 2005. Nitric oxide synthases II and III and vascular endothelial growth factor are up-regulated in sciatic nerve after spiral cuff electrode implantation. J Neuroimmunol 166:158–166.

Thomsen LL, Miles DW, Happerfield L, Bobrow LG, Knowles RG, Moncada S. 1995. Nitric oxide synthase activity in human breast cancer. Br J Cancer 72:41–44.

Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. 2007. Ganglioside GM2-tetraspanin CD82 complex inhibits met and its cross-talk with integrins, providing a basis for control of cell motility through glycosynapse. J Biol Chem 282:8123–8133.

Todeschini AR, Dos Santos JN, Handa K, Hakomori SI. 2008. Ganglioside GM2/GM3 complex affixed on silica nanospheres strongly inhibits cell motility through CD82/cMet-mediated pathway. Proc Natl Acad Sci USA 105:1925–1930.

Wang L, Takaku S, Wang P, Hu D, Hyuga S, Sato T, Yamagata S, Yamagata T. 2006. Ganglioside GD1a regulation of caveolin-1 and Stim1 expression in mouse FBJ cells: Augmented expression of caveolin-1 and Stim1 in cells with increased GD1a content. Glycoconj J 23:303–315.

Wang L, Wang Y, Sato T, Yamagata S, Yamagata T. 2008. Ganglioside GD1a suppresses TNFalpha expression via Pkn1 at the transcriptional level in mouse osteosarcoma-derived FBJ cells. Biochem Biophys Res Commun 371:230–235.

Wright B, Lacchini AH, Davies AJ, Walker AJ. 2006. Regulation of nitric oxide production in snail (Lymnaea stagnalis) defence cells: A role for PKC and ERK signalling pathways. Biol Cell 98:265–278.

Xie QW, Cho HJ, Calaycay J, Mumford RA, Swiderek KM, Lee TD, Ding A, Troso T, Nathan C. 1992. Cloning and characterization of inducible nitric oxide synthase from mouse macrophages. Science 256:225–228.

Yamagata S, Ito Y, Tanaka R, Shimizu S. 1988. Gelatinases of metastatic cell lines of murine colonic carcinoma as detected by substrate-gel electrophoresis. Biochem Biophys Res Commun 151:158–162.

Ziche M, Morbidelli L, Choudhuri R, Zhang HT, Donnini S, Granger HJ, Bicknell R. 1997. Nitric oxide synthase lies downstream from vascular endothelial growth factor-induced but not basic fibroblast growth factor-induced angiogenesis. J Clin Invest 99:2625–2634.