Biochemical and Biophysical Research Communications 419 (2012) 7–13

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

pp-GalNAc-T13 induces high metastatic potential of murine Lewis lung cancer by generating trimeric Tn antigen

Yasuyuki Matsumoto [a], Qing Zhang [a], Kaoru Akita [b], Hiroshi Nakada [b], Kazunori Hamamura [a], Noriyo Tokuda [a], Akiko Tsuchida [a,c], Takeshi Matsubara [a], Tomoko Hori [a], Tetsuya Okajima [a], Keiko Furukawa [a,d], Takeshi Urano [e], Koichi Furukawa [a,*]

[a] Department of Biochemistry II, Nagoya University Graduate School of Medicine, 65 Tsurumai, Showa-ku, Nagoya 466-0065, Japan
[b] Department of Molecular Biosciences, Faculty of Life Sciences, Kyoto Sangyo University, Kamigamo-Motoyama, Kita-ku, Kyoto 603-8555, Japan
[c] Noguchi Institute, 1-8-1 Kaga, Itabashi, Tokyo 173-0003, Japan
[d] Department of Biomedical Sciences, Chubu University College of Life and Health Sciences, 1200 Matsumoto-cho, Kasugai 487-8501, Japan
[e] Department of Biochemistry, Shimane University School of Medicine, Izumo 693-8501, Japan

ARTICLE INFO

Article history:
Received 15 January 2012
Available online 28 January 2012

Keywords:
Metastasis
pp-GalNAc-T
GM1
Invasion
O-glycan
Lung cancer

ABSTRACT

In order to analyze the mechanisms for cancer metastasis, high metastatic sublines (H7-A, H7-Lu, H7-O, C4-sc, and C4-ly) were obtained by repeated injection of mouse Lewis lung cancer sublines H7 and C4 into C57BL/6 mice. These sublines exhibited increased proliferation and invasion activity *in vitro*. Ganglioside profiles exhibited lower expression of GM1 in high metastatic sublines than the parent lines. Then, we established GM1-Si-1 and GM1-Si-2 by stable silencing of GM1 synthase in H7 cells. These GM1-knockdown clones exhibited increased proliferation and invasion. Then, we explored genes that markedly altered in the expression levels by DNA microarray in the combination of C4 vs. C4-ly or H7 vs. H7 (GM1-Si). Consequently, *pp-GalNAc-T13* gene was identified as up-regulated genes in the high metastatic sublines. Stable transfection of *pp-GalNAc-T13* cDNA into C4 (*T13*-TF) resulted in increased invasion and motility. Then, immunoblotting and flow cytometry using various antibodies and lectins were performed. Only anti-trimeric Tn antibody (mAb MLS128), showed increased expression levels of trimeric Tn antigen in *T13*-TF clones. Moreover, immunoprecipitation/immunoblotting was performed by mAb MLS128, leading to the identification of an 80 kDa band carrying trimeric Tn antigen, i.e. Syndecan-1. Stable silencing of endogenous pp-GalNAc-T13 in C4-sc (*T13*-KD) revealed that primary tumors generated by subcutaneous injection of *T13*-KD clones showed lower coalescence to fascia and peritoneum, and significantly reduced lung metastasis than control clones. These data suggested that high expression of *pp-GalNAc-T13* gene generated trimeric Tn antigen on Syndecan-1, leading to the enhanced metastasis.

1. Introduction

Metastasis is a major cause of death in human cancers. Mechanisms for the acquisition of metastatic potential are, however, not well understood. Cancer metastasis includes many processes such as invasion into surrounding tissues, release from the primary tumors, intravasation, adhesion to vascular walls, extravasation, and formation of new foci. Consequently, multiple factors are involved in individual steps of metastasis. In the past decade, a number of molecules involved in the metastasis have been identified, and their mutual correlations have also been investigated [1]. They were involved in the cell adhesion [2], angiogenesis [1,3], lymphangiogenesis [4,5], and protease-mediated migration and invasion [6].

We previously found that ganglioside GM1 was down-regulated specifically in high metastatic sublines of mouse Lewis lung cancer by repeated intravenous or subcutaneous injection [7]. In this study, we searched up- or down-regulated genes in the combination of C4 vs. C4-ly or H7 vs. H7 GM1-knockdown lines. Consequently, *pp-GalNAc-T13* gene was identified as an up-regulated gene in high metastatic sublines. Among 15 pp-GalNAc-T members identified in mammal so far [8,9], *pp-GalNAc-T13* gene was highly and exclusively expressed in the brain and present at very low or undetectable levels in other tissues [10].

Here, we demonstrated that a unique *O*-glycan structure, trimeric Tn (tTn) antigen, a structure consisting of three consecutive GalNAc-substituted amino acids (serine or threonine), generated by pp-GalNAc-T13 might play critical roles in cancer metastasis.

* Corresponding author. Fax: +81 52 744 2069.
E-mail address: koichi@med.nagoya-u.ac.jp (K. Furukawa).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2012.01.086

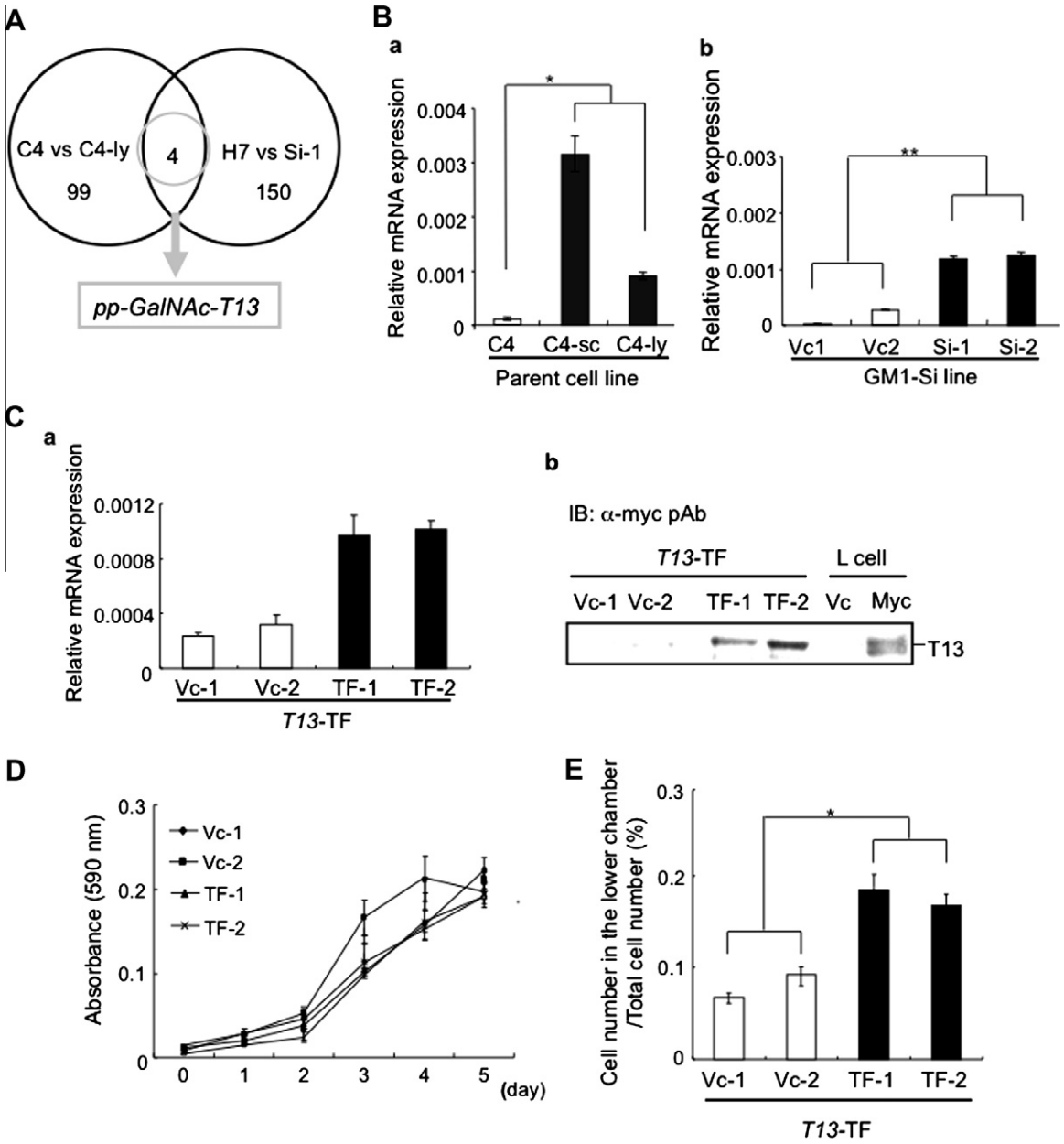

Fig. 1. Gene expression profiles and phenotypes of stable transfectant lines of *pp-GalNAc-T13*. Results of DNA microarray. (A) *pp-GalNAc-T13* gene was identified as one of up-regulated genes in the high metastatic sublines, i.e. C4-ly and H7 (GM1 synthase-knockdown line, Si-1). (B) Expression levels of *pp-GalNAc-T13* gene were confirmed by real time RT-PCR (a, C4 cell lines; b, GM1 synthase-knockdown lines). Bars represent ± S.D. ($n = 3$). (C) Transfection of *pp-GalNAc-T13* cDNA in pCMV3B expression vector into C4 resulted in the establishment of stable transfectant lines (TF-1 and TF-2). Vector control lines (Vc-1 and Vc-2) were also generated. Expression levels of *pp-GalNAc-T13* confirmed by real time RT-PCR (a) and immunoblotting (b). Bars represent ± S.D. ($n = 3$). L cell was a control. (D) Proliferation was examined by MTT assay. Bars represent ± S.D. ($n = 3$). (E) Invasion assay, in which 5×10^5 cells were seeded in the upper chamber in the absence of serum. After 24 h, invaded cell numbers were counted. Columns represent means ± S.D. ($n = 3$). A representative of three independent experiments was shown. $^{*}P < 0.01$; $^{**}P < 0.001$.

2. Materials and methods

2.1. Antibodies and lectins

Various lectins (AAL, PNA, RCA120, SBA, and SSA) were purchased from Seikagaku Kogyo (Tokyo, Japan). Mouse anti-Tn antibody (Bric111) was from Acris GmbH (Acris, Germany). Mouse anti-sialyl Tn antibody (B35.1) was from Abcam. Mouse anti-T antibody (A78-G/A7) was from Gene Tex, Inc. Mouse anti-Le^a antibody (7LE), mouse anti-Le^b antibody (2–25LE), mouse anti-Le^x antibody (73–30), mouse anti-Le^y antibody (H18A) and mouse anti-sialyl Le^a antibody (1H4) were purchased from Seikagaku Kogyo. Mouse anti-trimeric Tn (tTn) antibody (MLS128) was generated by Nakada et al. [11]. Rabbit anti-myc antibody (A-14) and rabbit anti-Syndecan-1 antibody (H-174) were purchased from Santa cruz Biotechnology (Santa cruz, CA). Mouse anti-sialyl Le^x antibody was from BD Transduction Laboratories (San Jose, CA). Cholera toxin B (CTB) subunit-biotin conjugates was from List Biological Laboratories, Inc. (Campbell, CA). Anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) was purchased from Amersham Bioscience. Anti-rabbit IgG antibody conjugated with HRP was from Cell Signaling Technology (Beverly, MA). FITC-labeled anti-mouse IgG antibody was purchased from ICN/Cappel (Durham, NC). FITC-labeled streptavidin was from EY Laboratories, Inc. (San Mateo, CA). Anti-mouse IgG conjugated with HRP (Mouse TrueBlot™ ULTRA) was from Bay bioscience. Anti-rabbit

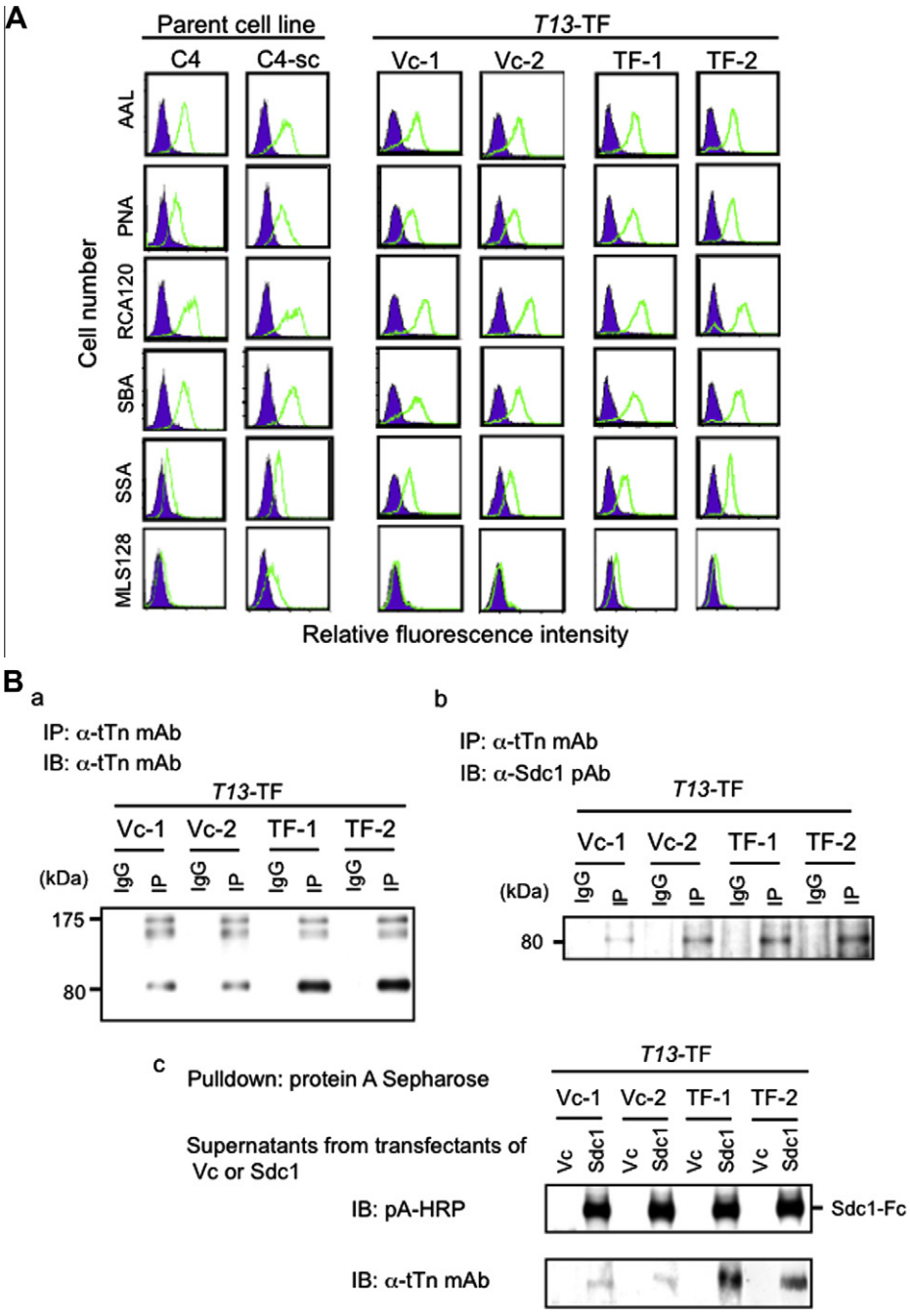

Fig. 2. *pp-GalNAc-T13* gene expression resulted in the formation of trimeric Tn antigen on Syndecan-1. (A) Expression levels of cell surface glycoconjugates were analyzed by flow cytometry with various lectins and anti-trimeric Tn (tTn) mAb (MLS128). (B) Cells were solubilized in the lysis buffer containing Triton X-100, and the lysates were immunoprecipitated with 2 μg of anti-trimeric Tn (tTn) mAb or normal mouse IgG at 4 °C. After SDS–PAGE of the immunoprecipitates with an anti-trimeric Tn mAb, immunoblotting was performed with each Ab (a: anti-tTn mAb, b: anti-Syndecan-1 pAb). IgG, normal mouse IgG; IP, immunoprecipitation with anti-tTn mAb. A representative result out of three independent experiments was shown. Syndecan-1-Fc (Sdc1-Fc) was expressed in *T13*-TF cells, and the supernatants were used for pulldown with protein A Sepharose. tTn bands were detected in TF-1/2-derived Sdc1-Fc by IB with anti-tTn mAb (c).

IgG antibody conjugated with Alexa488 was purchased from Invitrogen.

2.2. Cell lines and culture

Establishment of high metastatic sublines (C4-ly; lymph node, C4-sc; lung) from Lewis lung cancer cell line (sublines) H7 and C4 was as described [7]. These sublines were then maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. Stable transfectant cells of *pp-GalNAc-T13* gene (*T13*-TF) and control cells were cultured in DMEM supplemented with 7.5% FBS and G418 (350 μg/ml). Stable knockdown cells of *pp-GalNAc-T13* gene (*T13*-KD) and controls were cultured in DMEM supplemented with 7.5% FBS and blasticidin (2 μg/ml). GM1 synthase RNAi transfectant (GM1-Si) cells and control cells were cultured in DMEM supplemented with 7.5% FBS and puromycin (6 μg/ml) (Calbiochem).

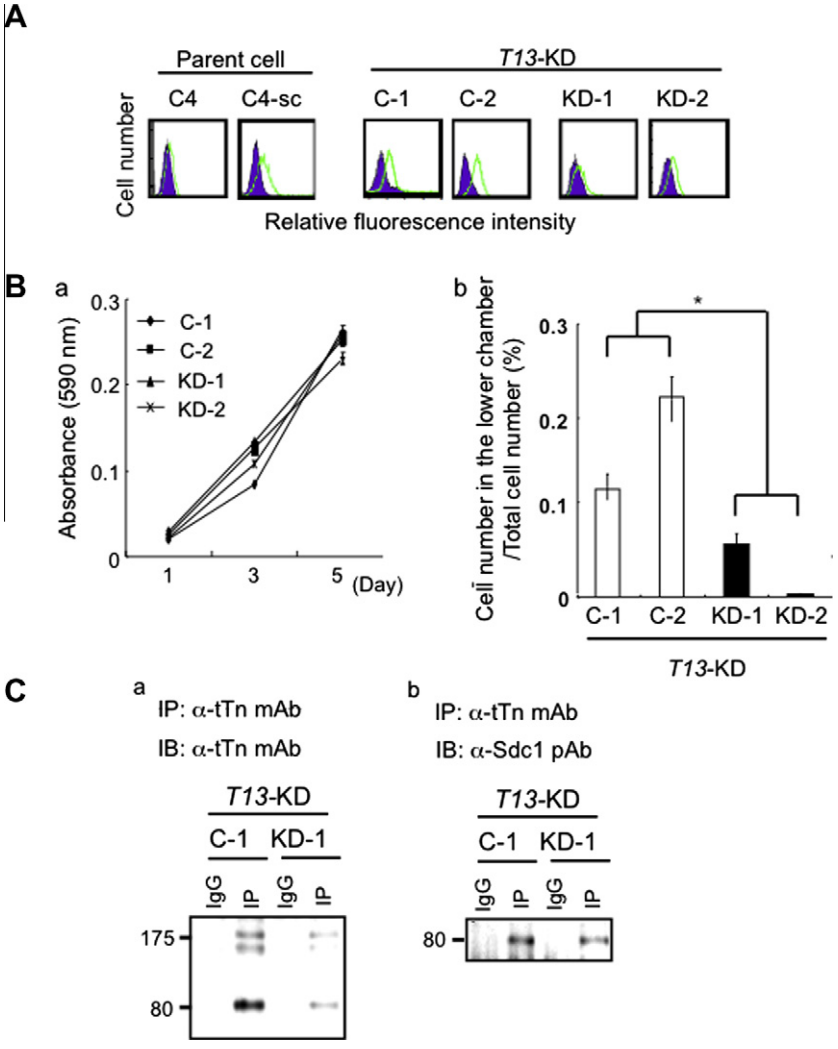

Fig. 3. Knockdown of *pp-GalNAc-T13* resulted in the reduced invasion and tTn on Syndecan-1. Transfection of *pp-GalNAc-T13* shRNA vector into C4-sc (*T13*-KD) resulted in the establishment of knockdown lines (KD-1 and KD-2). Vector control lines (C-1 and C-2) were also generated. (A) Expression levels of tTn antigen were analyzed by flow cytometry. (B) Proliferation (a) and invasion assay (b) were performed in *T13*-KD lines as described in Section 2. Bars represent ± S.D. ($n = 3$). Columns represent means ± S.D. ($n = 3$). $^{*}P < 0.01$. (C) Cell lysates were immunoprecipitated by IgG (normal mouse IgG) or anti-tTn mAb (IP), and immunoblotted using antibodies indicated in the figure. Note markedly reduced tTn antigen on Syndecan-1.

2.3. DNA microarray

The genome-wide gene expression profile of two pairs of parent and highly metastatic lines: C4 and C4-ly, and H7 and GM1-Si was analyzed using Affymetrix whole genome 430 2.0 GeneChips™ (Affymetrix, Santa Clara, CA). Detailed procedures were described in Supplemental materials and methods.

2.4. Assay of metastasis

Cells were detached from culture dishes by 5 min of treatment with 0.02% EDTA in PBS, centrifuged, and resuspended in 200 ml of PBS. For the spontaneous metastasis assay, cells (2×10^6/mouse) were inoculated subcutaneously into the thigh of age-matched female C57BL/6 mice (Nippon SLC, Hamamatsu, Japan). Mice were sacrificed at 4 weeks after injection, and metastatic nodules on the surface of lungs were counted by naked eye. Microscopic examination of tissues had been performed to confirm the identity of metastatic foci. The experimental metastasis assay was as described [7]. In brief, cells (1.0 or 0.5×10^6/mouse) were injected into the tail vein of C57BL/6 mice. Mice were sacrificed 4 weeks after injection, and metastatic nodules on the surface of lungs were counted. All mouse experiments were performed following the guideline of the Nagoya University Committee on Animal Research. When these guidelines were constructed, the "Principles of laboratory animal care" (NIH Publication No. 86-23, revised 1985) were followed as well as the guideline from the Ministry of Education, Culture, Sports and Technology of Japan (MEXT).

2.5. Real time RT-PCR

Total RNA was isolated in Trizol™ (Invitrogen), and template cDNA was synthesized from total RNA using reverse transcriptase kit (Invitrogen). Real time RT-PCR was done using DNA Engine Opticon3™ System (Bio-Rad). cDNA product (8 ng) was amplified in a 20-μl reaction containing 10 μl of DyNAmo™ SYBR Green qPCR kit (Finnzymes, Espoo, Finland) and 1 μl of each primer (5 μM). The primers for *pp-GalNAc-T13* were shown in Supplemental Table S1, and PCR program was shown in Supplemental materials and methods.

2.6. Construction of pp-GalNAc-T13 expression vector and transfection

Mouse *pp-GalNAc-T13* cDNA was generated by reverse transcription polymerase chain reaction (RT-PCR) using total RNA extracted from a C4-sc clone. The cDNA was digested with

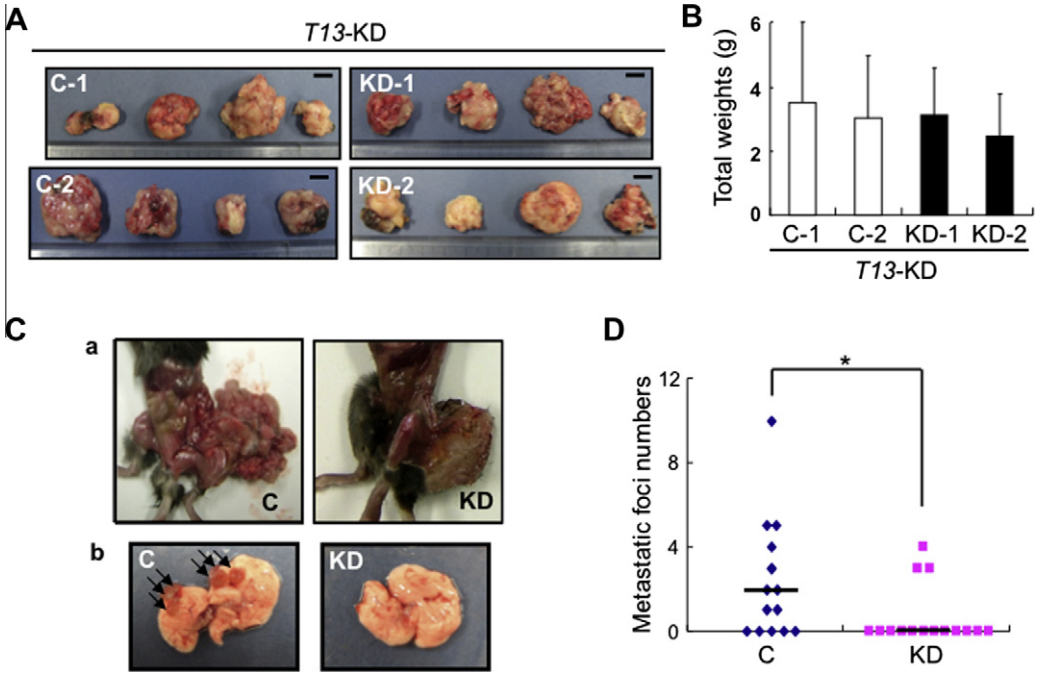

Fig. 4. Knockdown of *pp-GalNAc-T13* resulted in the reduced invasion and lung metastasis *in vivo*. (A) Examples of primary tumors with s.c. injection of *T13*-KD (a). Scale bar was 1 cm. (B) Weights of primary tumors were shown in a graph. Bars represent ± S.D. (C-1, KD-1; $n = 8$, C-2, KD-2; $n = 7$). (C) Primary tumors coalescent to fascia and peritoneum (a) and lung metastasis (b) in C (controls) were shown compared with no coalescence and intact lung of KD lines. Arrows indicate metastasis foci. (D) Whisker plots to show the distribution of the metastatic foci numbers. Bars represent the median value ($n = 15$). $^*P < 0.05$.

*Bam*HI-*Xho*I site of pCMV-Tag 3™ (Stratagene, North Turrey, CA), a mammalian expression vector with myc-tag (attached at N-terminus), to obtain pCMV-Tag 3/*pp-GalNAc-T13*. For stable transfection, this plasmid was transfected into C4 cells (30–40% confluent) in 6 cm culture dishes, and either 8 μg of pCMV-Tag 3/*pp-GalNAc-T13* or pCMV-Tag 3 alone was introduced using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions. After 24 h, 350 μg/ml of G418 (Invitrogen) was added to the culture medium to select G418-resistant clones. Two weeks later, independent colonies were picked up, and tested for expression levels of *pp-GalNAc-T13* by real time RT-PCR and western immunoblotting. The selected stable clones with increased levels of *pp-GalNAc-T13* were maintained in a complete culture medium containing G418 (350 μg/ml).

2.7. Expression vector of RNAi against pp-GalNAc-T13 gene and transfection

The RNAi expression vectors were constructed using an expression vector pcDNA™ 6.2-GW/EmGFP-miR (Invitrogen), which contained a DNA template for the synthesis of pre-miRNA ds oligo. The 21-mer candidate target sequences were selected from the open reading frame of *pp-GalNAc-T13* (GenBank™ accession number NM_173030), corresponding to nucleotides 2076–2096 (relative to the start codon) of *pp-GalNAc-T13*. The pre-miRNA ds oligo sequence contained 5-mer sequence for 5' overhang derived from miR-155, 21-mer target sequences, a 19-mer spacer, another 19-mer reverse complementary target sequence. The full-length of the sequence was 64 nucleotides, which were synthesized in the forward and reverse directions and annealed to form double-stranded DNA. This double-stranded DNA was cloned into pcDNA™ 6.2-GW/EmGFP-miR to establish pcDNA™ 6.2-GW/EmGFP-miR/*pp-GalNAc-T13*. For stable transfection of the RNAi expression vector, either 8 μg of pcDNA™ 6.2-GW/EmGFP-miR/*pp-GalNAc-T13* or pcDNA™ 6.2-GW/EmGFP-miR alone was introduced into C4-sc cells (30–40% confluent) in 6-cm dishes using Lipofectamine 2000™ (Invitrogen). After 24 h, 2 μg/ml of blasticidin (Calbiochem) was added to the culture medium to select blasticidin-resistant clones. Two weeks later, independent colonies were picked up, and tested for expression levels of *pp-GalNAc-T13* by real time RT-PCR. The selected clones with decreased levels of *pp-GalNAc-T13* were maintained in a complete culture medium containing blasticidin (2 μg/ml).

2.8. Flow cytometric analysis

Flow cytometry of cell surface gangliosides and other antigens was performed as described [7]. Procedures were described in Supplemental materials and methods.

2.9. MTT assay

Cells were seeded into 96-well plates and MTT assay was performed as described [7]. Procedures were described in Supplemental materials and methods.

2.10. Matrigel invasion assay

Cells (0.5×10^6/well) were seeded in the upper chamber of Matrigel-coated transwell filters (BD Biosciences). The DMEM containing 7.5% FBS was added to the lower chamber. Procedures were as described [7] and in Supplemental materials and methods.

2.11. Cell lysis and Western immunoblotting

Cells (0.5×10^6/dish) were seeded in 6-cm culture dishes and cultured in DMEM containing 7.5% FBS. Cell lysis and Western immunoblotting were performed as described [7]. Procedures were described in Supplemental materials and methods.

2.12. Statistical analysis

Statistical significance of data was determined by using Student's *t*-test.

3. Results

3.1. pp-GalNAc-T13 was identified as an up-regulated gene in C4-ly and in Si-1

In previous report, we established high metastatic sublines of Lewis lung carcinoma with *in vivo* selection strategy [7]. From two Lewis lung cancer line, H7 and C4, we established high metastatic sublines (C4-ly; lymph node metastasis of C4 by s.c. injection, C4-sc; lung metastasis of C4 by s.c. injection) by repeating injection of them into C57BL/6 mice with intravenous (i.v.) or subcutaneous (s.c.) injection (Supplemental Fig. S1). They exhibited increased cell proliferation and invasion activity *in vitro* (Supplemental Fig. S2). In addition, ganglioside profiling revealed low expression levels of GM1 in high metastatic sublines [7]. Then, we established GM1 synthase-knockdown lines in H7 (Si-1 or -2). Vector controls (Vc-1 or -2) were also generated. GM1 synthase-knockdown lines (GM1-Si) showed increased cell proliferation and invasion activity *in vitro* [7]. Then, we explored genes that markedly altered in their expression levels with DNA microarray. As up-regulated genes, 99 (C4 vs. C4-ly) genes and 150 (Vc-1 vs. Si-1) genes were identified. As a glycosyltransferase or related genes, *pp-GalNAc-T13* gene was identified among up-regulated genes (in C4-ly and Si-1) (Fig. 1A). Expression levels of *pp-GalNAc-T13* gene were confirmed by real time RT-PCR in C4 cell lines and GM1si lines (Fig. 1B). These data suggested that *pp-GalNAc-T13* gene might be involved in the metastatic potential. By the way, other *pp-GalNAc-T* genes showed no difference between C4 and C4-sc except Galnt1 and 7. These two gene products have been reported to hardly synthesize tTn structure (Supplemental Fig. S3).

3.2. Transfectant lines of pp-GalNAc-T13 cDNA exhibited increased invasion activity

To examine whether *pp-GalNAc-T13* gene is involved in cancer metastasis, we established stable transfectant lines (*T13*-TF; TF-1 and TF-2) using C4. Vector control lines (Vc-1 and Vc-2) were also generated. Expression of *pp-GalNAc-T13* was confirmed by real time RT-PCR (Fig. 1C(a)) and immunoblotting (Fig. 1C(b)). Actually, *T13*-TF exhibited increased invasion activity, while cell growth showed no differences (Fig. 1D and E). These data suggested that glycoconjugates modulated by pp-GalNAc-T13 affected invasion activity.

3.3. High expression of pp-GalNAc-T13 gene resulted in the formation of tTn antigen on Syndecan-1

To investigate carbohydrate structures on cell surface in C4, C4-sc and their derivatives, we performed flow cytometry with various anti-carbohydrate antibodies. Unexpectedly, unique carbohydrate structures such as sLe^X, which is involved in cancer metastasis, were not expressed on C4 and C4-sc cells (Supplemental Fig. S4). Glycoconjugate expression in *T13*-TF showed little differences from the parent cells (Fig. 2A). Since It has been reported that pp-GalNAc-T13 enzyme formed tTn antigen on mucin-like proteins [10], we examined the expression of tTn antigen using an anti-tTn antibody (MLS128), showing increased expression levels of tTn antigen in *T13*-TF clones as well as C4-sc (Fig. 2A). Then, immunoprecipitation/immunoblotting was performed to analyze carrier proteins of tTn antigen. Three bands (80, 150, 175 kDa) were detected as molecules carrying tTn antigen (Fig. 2B(a)). The identity of the strongest 80 kDa band was clarified as Syndecan-1 (Fig. 2B(b)). Syndecan-1-Fc was also expressed in *T13*-TF cells to verify Syndecan-1 really bears tTn using the culture supernatants. Definite tTn bands were detected in TF-1/2-derived Syndecan1-Fc (Fig. 2B(c)). Expression levels of Syndecan-1 in *T13*-TF cells were not changed (Supplemental Fig. S5), suggesting that high expression of *pp-GalNAc-T13* gene resulted in just formation of tTn antigen on Syndecan-1, leading to high metastasis.

3.4. Silencing of pp-GalNAc-T13 in C4-sc resulted in the reduction of tTn antigen expression and of invasion activity

To examine whether endogenous *pp-GalNAc-T13* gene is involved in cancer metastasis, we established stable silenced lines using C4-sc (*T13*-KD; KD-1 and KD-2). Vector control lines (C-1 and C-2) were also generated. As expected, *T13*-KD clones showed reduced expression levels of tTn antigen (Fig. 3A). *T13*-KD showed reduced invasion activity, though cell growth was not different (Fig. 3*B*). Correspondingly, *T13*-KD clones showed lower tTn antigen on Syndecan-1 (Fig. 3C).

3.5. Silencing of pp-GalNAc-T13 resulted in the reduced invasion and metastasis in mouse experiments

Metastasis experiments were performed using *T13*-KD clones. Although there were no differences in primary tumor weights at the injection sites (Fig. 4A and B), tumors derived from *T13*-KD clones (KD1 and KD2) showed poor coalescence to fascia and peritoneum compared with controls (Fig. 4C(a)). *T13*-KD clones showed significantly reduced lung metastasis *in vivo* (Fig. 4C(b) and D). These data suggested that tTn antigen generated on Syndecan-1 molecules by *pp-GalNAc-T13* gene plays a critical role in enhancement of cell invasion and cancer metastasis.

4. Discussion

A large fraction of cellular protein *O*-glycosylation is directed to producing a series of core-type *O*-glycan structures that begin with the covalent linkage of *N*-acetylgalactosamine (GalNAc) to serine and threonine residues of proteins [12,13]. This initial enzymatic step by a polypeptide-GalNAc transferases (pp-GalNAc-Ts) is followed by the regulated attachment of other sugars to this GalNAc residue by other glycosyltransferases in the Golgi apparatus, contributing to the changes of *O*-glycan repertoire in a given cell [14,15]. To date, 15 distinct pp-GalNAc-T members have been identified in mammal [8,9]. *pp-GalNAc-T13* gene was highly and exclusively expressed in the brain and at very low or undetectable levels in other tissues [10]. pp-GalNAc-T13 was able to form a tTn epitope, three consecutive GalNAc-Ser/Thr structures [10]. Tn-antigen and T-antigen derived from *O*-glycan are antigens associated with carcinomas and are generally masked by covalently linked terminal carbohydrate moieties in normal human tissues, but are exposed in most primary and metastatic epithelial malignant tumors [16–18]. Thus, tTn antigen might be more specific and promising as a tumor marker.

In order to analyze the mechanisms for cancer metastasis, DNA microarray was performed in the combination of C4 vs. C4-ly or H7 vs. H7 (GM1-si). Consequently, we identified four genes commonly up-regulated in the high metastatic groups. Among them, we identified *pp-GalNAc-T13* gene as the candidate gene responsible for the enhanced metastasis (Fig. 1A). The expression levels of *pp-GalNAc-T13* gene were supposed to be regulated by GM1 via unknown factors downstream of GM1-mediated signaling pathway. Despite rigorous trials to detect the differences in carbohydrate

expression using various carbohydrate-recognizing antibodies and lectins, no or little changes in *T13*-TF and -KD clones could be detected except for tTn antigen (Fig. 2, Supplemental Fig. S4). *pp-GalNAc-T13*-expressing cells (*T13*-TF) exhibited increased cell invasion activity (Fig. 1E), and *pp-GalNAc-T13*-silenced cells (*T13*-KD) showed decreased invasion and the suppression of metastatic potential (Figs. 3 and 4). These data suggested that the changes in *O*-glycans on cell surface might be involved in cancer malignancy, and tTn might be a critical molecule for the enhancement of malignant properties in cancer cells.

To date, it has been reported that pp-GalNAc-T1, 2 and 3 formed Tn antigen on MUC1. Another report described that MUC1 with sialyl Tn antigen is formed in human breast carcinomas [14,19]. Other studies reported that pp-GalNAc-T2 regulates invasion and metastasis of human glioma cells [20], or pp-GalNAc-T3 promotes pancreatic cancer cells growth [21]. MUC1 glycosylated by pp-GalNAc-T6 is involved in cytoskeletal regulation in human breast cancer cells [22]. On the other hand, there have been no reports on the role of pp-GalNAc-T13 in cancers except that *pp-GalNAc-T13* gene was up-regulated in bone marrow of stage 4 neuroblastoma patients [23]. In this study, we first found that a unique carbohydrate structure, tTn antigen is synthesized on Syndecan-1, and might be involved in cancer metastasis.

Metastasis experiments with *pp-GalNAc-T13*-silenced cells (*T13*-KD) showed that lung metastatic foci significantly reduced *in vivo,* while tumor sizes at primary sites were not different (Fig. 4). In the past studies on *O*-glycan-lacking mice, very serious phenotypes have been reported. But sometimes not so serious. Core 1 structure knockout mice, for example, showed high lethality during embryo and neonatal stages [24,25]. On the other hand, knockout mice of *pp-GalNAc-T13* gene were not lethal and not different from wild type mice (unpublished data). Taken together with our results, it might be suggested that a gene therapy with RNAi for *pp-GalNAc-T13*-expressing carcinomas is very promising.

Molecular mechanisms by which tTn antigen on Syndecan-1 enhances metastatic potential are not clear at this moment. Effects of tTn modification on the functions of carrier proteins need to be clarified as shown in a death receptor [26]. Furthermore, interacting molecules with the tTn structure, if present, remain to be investigated, and it seems to be essential for the clinical application of tTn antigen/antibody system.

Acknowledgments

We thank T. Mizuno and Y. Nakayasu for technical assistance. This study was supported by a Grant-in-Aid for Scientific Research (21390076), and partly by a Grant-in-Aid for Scientific Research (23590371) from MEXT.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2012.01.086.

References

[1] A. Harlozinska, Progress in molecular mechanisms of tumor metastasis and angiogenesis, Anticancer Res. 25 (2005) 3327–3333.
[2] R. Kannagi, Molecular mechanism for cancer-associated induction of sialyl Lewis X and sialyl Lewis A expression-The Warburg effect revisited, Glycoconj. J. 20 (2004) 353–364.
[3] L.M. Ellis, Angiogenesis and its role in colorectal tumor and metastasis formation, Semin. Oncol. 31 (2004) 3–9.
[4] S.D. Nathanson, Insight into the mechanisms of lymph node metastasis, Cancer 98 (2003) 413–423.
[5] Y. Cao, Opinion: emerging mechanisms of tumour lymphangiogenesis and lymphatic metastasis, Nat. Rev. 5 (2005) 735–743.
[6] M. Bjorklund, E. Koivunen, Gelatinase-mediated migration and invasion of cancer cells, Biochim. Biophys. Acta 1755 (2005) 37–69.
[7] Q. Zhang, K. Furukawa, H.H. Chen, T. Sakakibara, T. Urano, K. Furukawa, Metastatic potential of mouse Lewis lung cancer cells is regulated via ganglioside GM1 by modulating matrix metalloprotease-9 localization in lipid rafts, J. Biol. Chem. 281 (2006) 18145–18155.
[8] E. Tian, E.K.G. Ten Hagen, Recent insights into the biological roles of mucin-type O-glycosylation, Glycoconj. J. 26 (2009) 325–334.
[9] E.P. Bennett, U. Mandel, H. Clausen, T.A. Gerken, T.A. Fritz, L.A. Tabak, Control of mucin-type O-glycosylation - a classification of the polypeptide galnac-transferase gene family. Glycobiology (2011) in press. [Epub ahead of print].
[10] Y. Zhang, H. Iwasaki, H. Wang, T. Kudo, T.B. Kalka, T. Hennet, T. Kubota, L. Cheng, N. Inaba, M. Gotoh, A. Togayachi, J. Guo, H. Narimatsu, Cloning and characterization of a new human UDP-*N*-acetyl-alpha-D-galactosamine: polypeptide *N*-acetylgalactosaminyl-transferase, designated pp-GalNAc-T13, that is specifically expressed in neurons and synthesizes GalNAc alpha-serine/threonine antigen, J. Biol. Chem. 278 (2003) 573–584.
[11] Y. Numata, H. Nakada, S. Fukui, H. Kitagawa, K. Ozaki, M. Inoue, T. Kawasaki, I. Funakoshi, I. Yamashita, A monoclonal antibody directed to Tn antigen, Biochem. Biophys. Res. Commun. 170 (1990) 981–985.
[12] P. Van den Steen, P.M. Rudd, R.A. Dwek, G. Opdenakker, Concepts and principles of O-linked glycosylation, Crit. Rev. Biochem. Mol. Biol. 33 (1998) 151–208.
[13] Y. Wang, N. Agrwal, A.E. Eckhardt, R.D. Stevens, R.L. Hill, The acceptor substrate specificity of porcine submaxillary UDP-GalNAc: polypeptide *N*-acetylgalactosaminyltransferase is dependent on the amino acid sequences adjacent to serine and threonine residues, J. Biol. Chem. 267 (1992) 12709–12716.
[14] I. Brockhausen, J.M. Yang, J. Burchell, C. Whitehouse, J. Taylor-Papadimitriou, Mechanisms underlying aberrant glycosylation of MUC1 mucin in breast cancer cells, Eur. J. Biochem. 233 (1995) 607–617.
[15] H. Schachter, I. Brockhausen, The biosynthesis of branched O-glycans, Symp. Soc. Exp. Biol. 43 (1989) 1–26.
[16] G.F. Springer, Immunoreactive T and Tn epitopes in cancer diagnosis, prognosis, and immunotherapy, J. Mol. Med. 75 (1997) 594–602.
[17] B.L. Wang, G.F. Springer, S.C. Carlstedt, Quantitative computerized image analysis of Tn and T (Thomsen-Friedenreich) epitopes in prognostication of human breast carcinoma, J. Histochem. Cytochem. 45 (1997) 1393–1400.
[18] J. Imai, M. Ghazizadeh, Z. Naito, G. Asano, Immunohistochemical expression of T, Tn and sialyl-Tn antigens and clinical outcome in human breast carcinoma, Anticancer Res. 21 (2001) 1327–1334.
[19] K.O. Lloyd, J. Burchell, V. Kudryashov, B.W. Yin, J. Taylor-Papadimitriou, Comparison of O-linked carbohydrate chains in MUC-1 mucin from normal breast epithelial cell lines and breast carcinoma cell lines. Demonstration of simpler and fewer glycan chains in tumor cells, J. Biol. Chem. 271 (1996) 33325–33334.
[20] J. Lui, L. Yang, M. Jin, L. Xu, S. Wu, Regulation of the invasion and metastasis of human glioma cells by polypeptide *N*-acetylgalactosaminyltransferase 2, Mol. Med. Report 4 (2011) 1299–1305.
[21] K. Taniuchi, R.L. Cerny, A. Tanouchi, K. Kohno, N. Kotani, K. Honke, T. Saibara, M.A. Hollingsworth, Overexpression of GalNAc-transferase GalNAc-T3 promotes pancreatic cancer cell growth, Oncogene 30 (2011) 4843–4854.
[22] J.H. Park, T. Nishidate, K. Kojima, T. Ohashi, K. Takegawa, T. Fujikane, K. Hirata, Y. Nakamura, T. Katagiri, Critical roles of mucin 1 glycosylation by transactivated polypeptide *N*-acetylgalactosaminyltransferase 6 in mammary carcinogenesis, Cancer Res. 70 (2010) 2759–2769.
[23] N. Berois, E. Blanc, H. Ripoche, X. Mergui, F. Trajtenberg, S. Cantais, M. Barrois, P. Dessen, B. Kågedal, J. Bénard, E. Osinaga, G. Raguénez, PpGalNAc-T13: a new molecular marker of bone marrow involvement in neuroblastoma, Clin. Chem. 52 (2006) 1701–1712.
[24] J. Fu, H. Gerhardt, J.M. McDaniel, B. Xia, X. Liu, L. Ivanciu, A. Ny, K. Hermans, R. Silasi-Mansat, S. McGee, E. Nye, T. Ju, M.I. Ramirez, P. Carmeliet, R.D. Cummings, F. Lupu, L. Xia, Endothelial cell O-glycan deficiency causes blood/lymphatic misconnections and consequent fatty liver disease in mice, J. Clin. Invest. 118 (2008) 3725–3737.
[25] Y. Wang, T. Ju, X. Ding, B. Xia, W. Wang, L. Xia, M. He, R.D. Cummings, Cosmc is an essential chaperone for correct protein O-glycosylation, Proc. Natl. Acad. Sci. USA 107 (2010) 9228–9233.
[26] K.W. Wagner, E.A. Punnoose, T. Januario, D.A. Lawrence, R.M. Pitti, K. Lancaster, D. Lee, M. von Goetz, S.F. Yee, K. Totpal, L. Huw, V. Katta, G. Cavet, S.G. Hymowitz, L. Amler, A. Ashkenazi, Death-receptor *O*-glycosylation controls tumor-cell sensitivity to the proapoptotic ligand Apo2L/TRAIL, Nat. Med. 13 (2007) 1070–1077.