In general, the carbohydrate structures of glycans attached to cell surface proteins change dramatically by malignant transformation. The most prominent change is the increment of highly branched N-glycans containing the GaLβ1→4GlcNAcβ1→6Man group, which is synthesized by N-acetylgalactosaminyltransferase (GlcNAcT) V and β4-galactosyltransferase (β4GalT). The increment of highly branched N-glycans is associated with malignant properties of cancer cells. On the other hand, the GlcNAcT V-deficient mice, in which the amounts of highly branched N-glycans are reduced, showed that the growth and metastasis of mammary tumor induced by polyoma virus middle T oncogene are suppressed. Therefore, the biosynthetic pathway of highly branched N-glycans is considered to be important for expressing the malignant properties of cancer cells. The gene expression levels of several glycosyltransferases including GlcNAcT IVA, GlcNAcT V, β4GalT1, β4GalT5, fucosyltransferase (FUT) I, FUT II, and α-2,6-sialyltransferase I have been shown to increase in cancer cells, and the changes in glycosylation induced by the altered expression levels of the glycosyltransferase genes are closely associated with the malignant properties of cancer cells. The GC-rich promoter, which lacks the TATA and CCAAT boxes, appears to be characteristic of mammalian glycosyltransferase genes, and the consensus binding sites for transcription factor Sp1 are found in the promoter regions of the glycosyltransferase genes. Sp1 belongs to the Sp transcription factor family containing zinc finger DNA binding domains, which bind to the GC box in the promoter and enhancer regions, and is involved in the regulation of many housekeeping genes. Since the expression of Sp1 has been reported to upregulate in various cancer cells such as lung, breast, gastric, and pancreatic cancer cells, the expression levels of some glycosyltransferase genes are considered to increase in cancer cells due to upregulation of Sp1-expression.

Our previous studies demonstrated that the malignant properties of cancer cells are suppressed by regulation of the expression levels of the β4GalT genes, indicating that the changes of cell surface glycosylation contribute to the suppression of malignant properties of cancer cells. Therefore, if Sp1-expression is downregulated by RNA interference (RNAi), not only the expression of cancer-related molecules but also glycosylation patterns can be changed in accordance with the altered gene expression of glycosyltransferases, which may lead to the suppression of malignant properties of cancer cells. In the present study, we established a stable clone whose Sp1-expression level was reduced by introducing small interfering RNA (siRNA) expression vector into A549 human lung cancer cell line, which contains large amounts of Sp1, investigated the cell surface protein glycosylation and malignant properties of the cells, and identified the glycosyltransferase gene responsible for the altered glycosylation by downregulation of Sp1.

**MATERIALS AND METHODS**

**Cell Line and Reagents** A549 human lung cancer cell line was cultured in Dulbecco’s modified Eagle’s medium
(DMEM) containing 10% fetal calf serum (FCS), 50 units/mL penicillin and 50 µg/mL streptomycin. Rabbit anti-human β-actin, anti-human Sp1, anti-human E-cadherin, and anti-human lysosome-associated membrane protein-1 (LAMP-1) antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated concanavalin A (Con A), *Ricinus communis* agglutinin-I (RCA-I), leuko-agglutinating phytohemagglutinin (L-PHA), and L-PHA-agarose were purchased from Seikagaku Kogyo (Tokyo). Mithramycin A was obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.).

**Plasmid Construction** For downregulation of Sp1 by RNAi, the target nucleotide sequences were selected from the human Sp1 gene (GenBank™ accession number: NM_138473) by siRNA Target Finder (Ambion, Austin, TX, U.S.A.). The uniqueness of each target sequence to the Sp1 mRNA was verified by BLAST analysis. The oligonucleotides, TSSP1 (5'-GAT CCT GGG GCC AAT GTT AAT GTT TTC AAG ACC ACG CTC AG-3') and TSSP2 (5'-AGC TTA ATG GGG GCA ATG GTA ATG GTT CTC TTG AAA ACC TTA CCA TTG GCC CCG CAG-3'), were designed and annealed for generating the double-stranded DNA fragment. The DNA fragment was ligated into the BamHI– HindIII sites of the pSilencer4.1-hygro vector (Ambion), to generate pSilencer/siSp1. As a control, pSilencer/Negative control vector (Ambion) was used.

Since the promoter region of the human β4GalT1 gene was identified, the promoter region (−500/−1 relative to the initiation codon) was synthesized, and ligated into pGL3-Basic vector (Promega, Madison, WI, U.S.A.), to generate pGL-GT1, pGL-GT1mSP1, and pGL-GT1mSP2. In order to generate three plasmids, pGL-GT1mSP1, pGL-GT1mSP2, and pGL-GT1mSP3, the mutations in the Sp1-binding sites were introduced using pGL-GT1 (−500/−1) and KOD-Plus-Mutagenesis Kit (TOYOBO, Osaka, Japan) according to the manufacturer’s instructions. The wild-type sequences of the Sp1-binding sites, GCCCCC GCCCC, at nucleotide positions −236/−227 and −213/−204 were replaced with GCCCGAACC, in which the mutations were underlined. pGL-GT1mSP1 and pGL-GT1mSP2 contain the mutation in the Sp1-binding sites −236/−227 and −213/−204, respectively. pGL-GT1mSP3 contains the mutations in both Sp1-binding sites. The correct sequences of plasmids with mutations were confirmed by nucleotide sequencing.

**Transfection** A549 cells were transfected with pSilencer/siSp1 and pSilencer/Negative control vector to establish the Sp1-downregulated cell clones and the control cell clone, respectively, as described previously. After being cultured for 24 h, the plasmid-transfected cells were selected with DEM containing 10% FCS and hygromycin B (500 µg/mL) for two weeks.

**Western Blotting** The control and Sp1-downregulated cells were homogenized, and then subjected to acetone precipitation as described previously. The defatted samples referred to as membrane glycoprotein samples were analyzed by Western blotting. Western blotting with Con A, RCA-I, L-PHA, or the antibody against Sp1, E-cadherin, or LAMP-1 was conducted as described previously. In the case of incubation with RCA-I and L-PHA, the filters were initially treated with 25 µM sulfuric acid at 80°C for 1 h. Lectin precipitation using L-PHA-agarose was performed as the method described previously. The intensity of Sp1 protein bands was determined, and the ratios between those of control and Sp1-downregulated cells were calculated as described previously.

**Quantitative Real-Time RT-PCR Analysis** Quantitative real-time RT-PCR analysis was conducted as the method described previously. The oligonucleotide primers specific to each gene were as follows: Sp1, F: 5'-GCCCTCAGCATTACCTCAG-3'; R: 5'-TCAAGCAGATCCATCCACCGAG-3'; β4GalT1, F: 5'-CGTGATCCAGTTAAGTTGGATCC-3'; β4GalT2, F: 5'-ACGGCGTCTAGTGCTCAACCAG-3'; β4GalT3, F: 5'-ACCCAAGGCTCCACTGAGAAG-3'; GlcNAcT V, F: 5'-GAGCAGATCTGAGACCTAGAC-3'; β4GalT2, F: 5'-ACGGCGTCTAGTGCTCAACCAG-3'; β4GalT3, F: 5'-ACCCAAGGCTCCACTGAGAAG-3'; β4GalT1, F: 5'-ACGGCGTCTAGTGCTCAACCAG-3'; β4GalT2, F: 5'-ACGGCGTCTAGTGCTCAACCAG-3'; β4GalT3, F: 5'-ACCCAAGGCTCCACTGAGAAG-3'.

**Luciferase Assay** The promoter activities of the reporter plasmids were determined by luciferase assay as described previously. Mithramycin A (0.1 µM) was added to A549 cells 1 h after transfection with pGL-GT1 (−500/−1) as described previously.

**Cell Proliferation Assay** Cells were seeded into 96-well plates at a density 1×10³ per well, and incubated for one to seven days. Growth rates of the control and Sp1-downregulated cells were examined using Celltiter 96 aqueous one solution cell proliferation assay (Promega) according to the manufacturer’s instructions. The absorbance of each well at 490 nm was measured using a microtiter plate reader.

**Anchorage-Independent Cell Growth Assay** Anchorage-independent cell growth assay was carried out as described previously. In 12-well tissue culture plates, 0.6% agarose was placed as bottom agarose, and then 2×10⁴ cells suspended in 0.3% agarose with DMEM containing 10% FCS were placed on the top of the bottom agarose. The plates were incubated for three weeks. The colony numbers (>100 µm in diameter) and colony sizes in five fields were measured under a microscope.

**Wound Healing Assay** Wound healing assay was performed as described previously. In brief, cells were seeded into 24-well tissue culture plate and grown to confluence. The cell monolayers were scratched with 200 µL yellow plastic tip, and then extensively washed with serum-free DMEM. The cells were incubated in DMEM containing 2% FCS and 100 µg/mL epidermal growth factor for the indicated time periods. Migration of the control and Sp1-downregulated cells into wound area was photographed with a microscope, and then cell migration distances were chronologically measured with a micrometer. The relative wound widths were expressed by taking the distance at the initial time as 1.0.

**Statistical Analysis** A significant difference was assessed by Student’s *t*-test.

**RESULTS**

**Establishment of Sp1-Downregulated Cell Clone** For downregulation of Sp1, the expression plasmid for siRNA against Sp1, pSilencer/siSp1, was transfected into A549 cells, and stable cell clones were obtained after selection with hygromycin B. One of the clones showed that the expression of the Sp1 gene decreases by 30% as compared to that of the control cell clone (Fig. 1A). Immunoblot analysis using an anti-Sp1 anti-
body showed that the expression of Sp1 decreases to approximately 50% in this clone when compared with the control cell clone (Fig. 1B), showing the highest level of Sp1-downregulation among the stable clones established. Since Sp1 is essential for various cellular functions including cell proliferation, the reason why the Sp1-downregulated cells with the expression level of Sp1 lower than 50% could not be obtained may be due to the dysfunction of cell proliferation. Therefore, in this study, we used the clone with the expression level of Sp1 reduced by 50% as the Sp1-downregulated cells.

**Decreased β4-Galactosylation of N-Glycans on E-Cadherin by Sp1-Downregulation**

Coomassie Brilliant Blue (CBB) staining showed that the membrane glycoprotein samples from the control and Sp1-downregulated cells appear similar protein components (lanes 1 and 2 of Fig. 2-CBB). To analyze whether or not the cell surface N-glycosylation changes in A549 cells by downregulation of Sp1, lectin blotting was conducted using Con A, RCA-I, and L-PHA. No significant change in the binding of Con A, which interacts mainly with high mannosetype N-glycans, was detected between the control and Sp1-downregulated cells (lanes 1 and 2 of Fig. 2-Con A), suggesting that the amounts of N-glycan sub-group are not changed by downregulation of Sp1. The filters were initially subjected to sulfuric acid treatment to remove sialic acid linked to galactose residues, and then incubated with RCA-I, which binds to glycans terminating with the N-GlcNAc group. A significant decrease in the lectin binding was observed for 76–86, 90, 94, 120, 145 and 160 kDa of protein bands in the Sp1-downregulated cells as compared with the control cells (lanes 1 and 2 of Fig. 2-RCA-I). Upon incubation with L-PHA, which recognizes specifically highly branched N-glycans with the Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man branch, 76–86, 90, 94 and 120 kDa of protein bands reacted weakly to the lectin in the Sp1-downregulated cells as compared with the control cells (lanes 1 and 2 of Fig. 2-L-PHA). The L-PHA-reactive protein bands were considered to correspond, most probably, to those bound to RCA-I as shown in Fig. 2-RCA-I. The carbohydrate-binding specificities of each lectin were confirmed by the methods as described previously.

When the filters were incubated with the antibodies, the 90 and 94 kDa of protein bands reacted with anti-LAMP-1 antibody, and the 120 kDa of protein band reacted strongly with anti-E-cadherin antibody (lanes 1 and 2 of Fig. 2-LAMP-1 and E-cadherin). LAMP-1 is a membrane glycoprotein that is predominantly expressed in lysosome. In this study, we focused on the 120 kDa of protein band, presumably E-cadherin, which is the well-characterized cell adhesion molecule. In order to identify the L-PHA-reactive 120 kDa of protein band, lectin precipitation using L-PHA followed by immunoblot analysis with anti-E-cadherin antibody was performed. The results showed that the L-PHA-reactive 120 kDa of protein band is identified as E-cadherin (Fig. 3). As shown in Fig. 2-E-cadherin, the expression of E-cadherin unchanged by downregulation of Sp1. On the other hand, the expression of LAMP-1 appeared to increase slightly in the Sp1-downregulated cells as compared with the control cells (Fig. 2-LAMP-1). Taken together, these results suggest that the β4-galactosylation of highly branched N-glycans on E-cadherin and LAMP-1 decreases by downregulation of Sp1.

**Reduced Expression of β4GalT1 Gene by Sp1-Downregulation**

The Galβ1→4GlcNAcβ1→6Man group in the highly branched N-glycans is synthesized by GlcNAcT V and β4GalTs. Although there are seven β4GalTs in mammalian cells, β4GalT1 and β4GalT2 are involved in the β4-galactosylation of N-glycans. To elucidate the background of the altered N-glycosylation by downregulation of Sp1, the expression levels of the β4GalT1, β4GalT2, and GlcNAcT V genes were analyzed by quantitative real-time RT-PCR. The results showed that the expression level of the β4GalT1 gene in the Sp1-downregulated cells decreases to 45% of the control cells (Fig. 4-β4GalT1). However, the expression levels of the β4GalT2 and GlcNAcT V genes unchanged between the control and Sp1-downregulated cells (Fig. 4-β4GalT2 and GlcNAcT V). These results strongly suggest that the decreased

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**Fig. 1.** The Expression Level of Sp1 in the Sp1-Downregulated Cells

(A) The expression levels of the Sp1 transcript in the control and Sp1-downregulated cells (Sp1-D) relative to those of the G3PDH transcript. Data show the means±S.D. (n=3). **p<0.01 against control. (B) Immunoblot showing expression of Sp1 protein in the control and Sp1-downregulated cells (Sp1-D). The ratio of amounts of Sp1 against β-actin between the control and Sp1-downregulated cells is shown at the bottom of the Sp1-blot.

**Fig. 2.** Western Blotting of Membrane Glycoproteins from the Control and Sp1-Downregulated Cells

The filters were incubated with CBB, Con A, RCA-I, L-PHA, anti-LAMP-1 antibody, and anti-E-cadherin antibody. Lanes 1 and 2 indicate the samples from the control and Sp1-downregulated cells, respectively. The arrow and arrowhead indicate the protein bands, which are reacted with anti-E-cadherin and anti-LAMP-1 antibodies, respectively.
β4-galactosylation of highly branched N-glycans in A549 cells by downregulation of Sp1 is brought about by the reduced expression of the β4GalT1 gene. Mithramycin A is well-known inhibitor for binding of Sp1 to its binding site in DNA. By the treatment of A549 cells with mithramycin A, the expression of the β4GalT1 gene decreased significantly (Fig. 5A), suggesting that the expression of the β4GalT1 gene is regulated by Sp1. To confirm this further, the promoter activity of the β4GalT1 gene was analyzed by luciferase assay. Two Sp1-binding sites were predicted at nucleotide positions −236/−227 (score: 94.5) and −213/−204 (score: 94.5) relative to the initiation codon in the β4GalT1 gene promoter by TFSEARCH program version 1.3 (http://www.cbrc.jp/research/db/TFSEARCH.html). We generated three constructs pGL-GT1mSP1, pGL-GT1mSP2, and pGL-GT1mSP3, in which the Sp1-binding sites were mutated (Fig. 5B). Our previous study clearly demonstrated that the similar mutation in the Sp1-binding site reduces the Sp1-binding to the promoter region, and leads to the decreased promoter activity of the human β4GalT5 gene. Upon transfection of these constructs into A549 cells, the luciferase activities decreased drastically when compared with the cells transfected with pGL-GT1 (−500/−1) (Fig. 5B). These results suggest that the Sp1-binding sites are critical and Sp1 plays pivotal roles in the promoter activity of the β4GalT1 gene.

**Suppression of Malignant Properties by Sp1-Downregulation.** The growth rate of the Sp1-downregulated cells was examined by cell proliferation assay. The results showed that in a growth phase, the growth rate of the Sp1-downregulated cells decreases by 17–31% compared to the control cells (Fig. 6), indicating that downregulation of Sp1 suppresses the cell growth. Generally, anchorage-independent cell growth in soft agar is correlated to tumorigenic potentials of cancer cells. Anchorage-independent cell growth assays showed that the average sizes of colonies formed by the control and Sp1-downregulated cells were 104.6±5.7 μm and 73.7±1.9 μm, respectively (Fig. 7A). In addition, the numbers of colonies over 100 μm in diameter formed by the control cells were 38±6.2 whereas those formed by the Sp1-downregulated cells were 10±0.8 (Fig. 7B). These results indicate that the anchorage-independent growth of A549 cells is reduced significantly by downregulation of Sp1. Dynamic cell movement is one of the characteristic features of cancer cells, and is related to metastasis. Next, the migratory activity of the Sp1-downregulated cells was examined by wound healing assay. The results showed that after 24h incubation, more than 80% of the wound area is filled in the control cells, but about 55% of the area is filled in the Sp1-downregulated cells (Fig. 8), indicating that the migratory activity decreases by downregulation of Sp1. Taken together, these results demonstrate that the malignant properties of A549 cells decrease by downregulation of Sp1.

**DISCUSSION**

The highly branched N-glycans have been shown to be involved in the malignant properties of cancer cells. In the present study, we established the Sp1-downregulated cells without apparent changes in protein components from human lung cancer cell line, and clearly demonstrated that by downregulation of Sp1 the β4-galactosylation of highly branched N-glycans on E-cadherin decreases (Fig. 2), and the malignant properties are suppressed (Figs. 6–8). Furthermore, we identified the β4GalT1 gene as the responsible gene for the decreased β4-galactosylation of N-glycans by Sp1-downregulation (Fig. 4). As human β4GalT1 has been shown to be involved in the β4-galactosylation of highly branched N-glycans, the binding of RCA-I and L-PHA, both of which interact with the β4-galactosylated N-glycans, decreased significantly in the Sp1-downregulated cells as the results of the reduced expression of the β4GalT1 gene (Figs. 2, 4). These results indicate that the biosynthesis of highly branched N-glycans is inhibited by downregulation of Sp1. Since the highly branched N-glycans have been shown to be important for malignant properties of cancer cells, the inhibition of the biosynthesis of highly branched N-glycans by Sp1-downregulation could partly contribute to the suppression of malignant properties of A549 cells with changes in the gene expression of cancer-related molecules. Moreover, sialylated glycans have been shown to be associated with malignant properties of cancer cells. Since the terminal galactose residues of glycans are often sialylated, the decreased β4-galactosylation of N-glycans by Sp1-downregulation is considered to bring about the...
decreased sialylation of N-glycans partially, which may also lead to the suppression of malignant properties of A549 cells. The expression of β4GalT1 has been shown to increase in highly metastatic human lung cancer cells, PGBE1, when compared with its less metastatic lung cancer cells, PGLH7. The migratory activity of PGBE1 was higher than that of PGLH7, and the invasive potential of PGBE1 decreased by reducing the expression of the β4GalT1 gene with RNAi. Therefore, the β4-galactosylated N-glycans to be synthesized by β4GalT1 could be involved in the malignant properties of lung cancer cells. In fact, we found that by reducing the expression of the β4GalT1 gene with RNAi, the anchorage independent growth of A549 cells is suppressed similar to the Sp1-downregulated cells (unpublished data). The decreased β4-galactosylation of highly branched N-glycans by Sp1-downregulation was mainly observed for E-cadherin (Figs. 2, 3). As the changes in N-glycosylation of E-cadherin have been shown to suppress the malignant properties of cancer cells by modulating the cell adhesion, the changes in N-glycosylation of E-cadherin could contribute to the suppression of the malignant properties of A549 cells. However, the decreased β4-galactosylation was also observed for LAMP-1, which was tentatively identified by immunoblot analysis (Fig. 2). Since LAMP-1 containing oversialylated glycans has been shown to enhance lysosomal exocytosis, which promotes the migration and invasion of human sarcoma cells, the changes in N-glycosylation of LAMP-1 could be involved in the suppression of the malignant properties. Although LAMP-1 is a lysosomal membrane glycoprotein, its cell surface expression is detected in small amounts. Therefore, the changes in N-glycosylation of LAMP-1 could contribute to the suppression of the malignant properties, too. The mechanism of the reduced malignant properties by down-regulation of Sp1 is currently unknown. Analysis of the signal transduction and expression of cancer-related molecules in the Sp1-downregulated cells will provide the further understanding of the molecular mechanism of the reduced malignant properties of A549 cells.

In mouse somatic tissues, the expression of the β4GalT1...
gene was regulated by a promoter having multiple Sp1-binding sites. Our results showed that the expression of the β4GalT1 gene decreases by the treatment with mithramycin A, which is an inhibitor for Sp1-binding, and the promoter activity decreases dramatically when the mutations were introduced in the Sp1-binding sites of the human β4GalT1 promoter region (Fig. 5B). These results indicate that Sp1 plays pivotal roles in the expression of the β4GalT1 gene. However, other transcription factors could also involve in the expression of the β4GalT1 gene since significant promoter activity was still detected though the mutations were introduced in both Sp1-binding sites (Fig. 5B). For instance, E1AF has been reported to activate the β4GalT1 gene promoter, which supports this possibility. On the other hand, the expression levels of the β4GalT2 and GlcNAcT V genes unchanged by downregulation of Sp1. Since the expression of the human β4GalT2 and GlcNAcT V genes has been shown to be regulated by p53 and Ets-1, respectively, downregulation of Sp1 could not affect to the expression levels of these genes.

This is the report, for the first time, showing that downregulation of Sp1 suppresses malignant properties of human lung cancer cell line in part due to the decreased β4-galactosylation of highly branched N-glycans. The reduced expression of the Sp1 gene in human fibrosarcoma cells by using Sp1 ribozyme antisense resulted in the suppression of tumorigenic potential with the decreased gene expression of vascular endothelial growth factor, which is one of the key molecules for tumor angiogenesis. By regulating the expression of transcription factors in cancer cells, the glycosyltransferases and cancer-related molecules can be regulated simultaneously, which may lead to the suppression of the malignant properties of cancer cells synergistically and effectively.

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Conflict of Interest The authors declare no conflict of interest.

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